A newly developed strain of *Enterococcus faecium* isolated from fresh dairy products to be used as a probiotic in lactating Holstein cows

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The objective of this study was to determine the ability of an isolated strain (EGY_NRC1) or commercial (NCIMB 11181) *Enterococcus faecium* as a probiotic for lactating cows. Two experiments were conducted: In Experiment 1, the effects of three levels (1, 2, and 3 g/kg diet, DM basis) of isolated and commercial *E. faecium* on *in vitro* ruminal fermentation kinetics, gas, methane (CH\(_4\)), and nutrient degradability were determined. In Experiment 2, thirty multiparous Holstein cows (633 ± 25.4 kg body weight) with 7 days in milk were randomly assigned to 3 treatments in a completely randomized design in a 60-day experiment. Cows were fed without any additives (control treatment) or supplemented with 2 g/kg feed daily of *E. faecium* EGY_NRC1 (contain 1.1 × 10\(^9\) CFU/g) or commercial *E. faecium* NCIMB 11181 (contain 2 × 10\(^{12}\) CFU/g). Diets were prepared to meet cows' nutrient requirements according to NRC recommendations. Probiotic doses were based on the *in vitro* Experiment 1. Feed intake, digestibility, blood parameters, and lactation performance were evaluated. In Experiment 1, the isolated *E. faecium* lineally and quadratically increased (\(P < 0.001\) in *in vitro* total gas production (TGP)), the degradability of dry matter (dDM) and organic matter (dOM) while decreased (\(P < 0.05\)) methane (CH\(_4\)) percent of TGP, NH\(_3\)-CH\(_4\) production, and pH. The commercial *E. faecium* increased TGP and decreased (\(P < 0.01\)) CH\(_4\) production, pH and increased the dDM and dOM, short chain fatty acids and ruminal NH\(_3\)-N concentration. In Experiment 2, the isolated *E. faecium* increased (\(P < 0.01\)) total tract digestibility of DM, neutral and acid detergent fiber, daily milk production and feed efficiency compared to the control treatment without affecting feed intake and milk composition. Moreover, the isolated *E. faecium* increased (\(P < 0.05\)) the proportion of C18:1 *trans*-9, C18:2 *cis*-9–12 and C18:2 *trans*-10 *cis*-12. Both isolated and commercial *E. faecium* improved (\(P < 0.01\)) organic matter, crude protein and nonstructural carbohydrates digestibility, increased serum glucose (\(P = 0.002\)) and decreased serum cholesterol (\(P = 0.002\)). Additionally, both
E. faecium strains decreased C23:0 ($P = 0.005$) in milk. In conclusion, the use of E. faecium (isolated and commercial) at 2 g/kg DM of feed improved feed efficiency and production performance, with superior effects on animal performance from isolated E. faecium compared to the commercial one.

**KEYWORDS**

Enterococcus faecium, digestion, feed utilization, lactic acid bacteria, milk production

**Introduction**

Probiotics and prebiotics have been administered to animals for several years to enhance their health and production. In animal production, probiotics are now widely accepted as safe and sustainable alternatives to antibiotics (1, 2). For many years, lactic acid bacteria (LAB) have been used in livestock production, as probiotic supplements or as silage preservative by inhibiting pathogenic microorganisms (e.g., fungal and clostridial growth) and increasing lactic acid formation (3, 4). Normally, LAB are predominantly found in the gastrointestinal tract of animals and humans and are also found in dairy products (5).

In livestock, different bacterial and fungal species (i.e., Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Propionibacterium, Megaphaera elsdenii and Prevotella bryantii) have been used as probiotics as well as strains of Aspergillus and Saccharomyces (1, 6). Strains of LAB including Lactobacillus, Bifidobacterium and Streptococcus are commonly used as probiotics in ruminant feed (1, 7). LAB can alter ruminal fermentation and enhance nutrient digestibility and productive performance (8). LAB reduce oxygen from the rumen environment and prevent excess of ruminal lactate production (5). LAB can alter ruminal fermentation and enhance nutrient digestibility and productive performance (8). LAB reduce oxygen from the rumen environment and prevent excess of ruminal lactate production (5).

To characterize the selected isolate, the carbohydrate fermentation pattern (14) of the selected isolate (that possessed antibacterial activity) and its ability to produce ammonia (NH₃) from arginine (15) was examined. After that, the strain was identified by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and 16S ribosomal DNA (rDNA) sequencing. The isolate was identified (Figure 1) via the analysis of its total proteome in which a score with more than 1.7 indicates genus identification and a score with more than 2 is the confidence value at the species level (16).

For DNA extraction, genomic DNA was used from the selected isolate that was cultivated at 37°C for 24 h. Freshly prepared culture was subjected to 16S rDNA PCR partial amplification by use of Qiagen genomic DNA purification kit. The genomic DNA was used as a template for PCR amplification of a segment of its 16S rRNA gene. The two primers used were previously described by Liu et al. (17), 8f (5_AAGAGTTTGATCCTGGCTCAG-3) and 1495 R (5_CTACGGGTACCGTGTAGCAG-3). The PCR products yielded were analyzed on a 1% (w/v) agarose gel after staining with ethidium bromide. The PCR products were separated on an agarose gel, followed by ethidium bromide staining to check for the presence of a unique amplicon. When a gene from a particular species was amplified using a primer initially designed for a different species, the corresponding amplicon was similar effects on feed utilization, milk production, milk composition and milk fatty acid profile in early lactating dairy Holstein cows.

**Materials and methods**

**Isolation and identification of E. faecium**

Lactic acid bacterial strains were isolated from 15 samples of fresh dairy products (homemade 8 samples of yogurt and 5 samples soft white cheese). For each sample, 10 g were added to 90 ml sterile saline solution and homogenized by vortex for 10 min. Decimal dilutions were placed on double layered M17 agar plates then incubated for 48 h at 30°C. Well defined round colonies were selected randomly and only Gram-positive catalase-negative cocci were retained and stored in M17 broth for further experiments.

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FIGURE 1
Result of the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The MALDI-TOF MS showed that the isolated strain of *E. faecium* that possess antimicrobial activity was initially identified with a high confidence value of 2.31 that indicated a reliable identification of the isolate up to species level.

purified by Promega Wizard Genomic DNA Purification Kit and sequenced by HVD life science, Germany.

For phylogenetic analysis (data not shown), the 16S rDNA sequence obtained was added to publicly available bacterial 16srRNA sequences and integrated into the database with the automatic alignment tool. Phylogenetic analysis was inferred using neighbor joining method and the phylogenetic tree was constructed (18). The isolated LAB was identified as *E. faecium* EGY_NRC1 with NCBI accession number MW856655.

The biomass production of the isolated LAB was done on whey permeate media with the following composition (/L permeate): 5 g yeast extract, 5 g peptone, 0.5 g magnesium sulfate, 3 g ammonium chloride and 2.5 g ascorbic acid. The medium was inoculated individually with 5% (v/v) of the isolated LAB culture (24 h old age activated M17 broth culture) and then incubated at 37°C for 48 h. The cultured biomasses were separated by centrifugation at 6,000 rpm for 15 min at 4°C then added to dry permeate as a carrier agent, and dried in oven at 37°C. The un-inoculated media were used as control. Unpublished data showed that the viable count of the strain after pre-incubation at 60°C for 30 min was enumerated and the result indicated that the remaining count was 3.274 log CFU/mL in compared to 5.477 log CFU/mL without pre-incubation. The reported viable count was determined after drying.

**Experiment 1**

**In vitro evaluation**

Using a stomach tube, rumen liquor was obtained from three adult Barki sheep (51 ± 2.6 kg of body weight) fed a fixed amount of concentrate (500 g) and *ad libitum* berseem hay daily. The rumen contents (liquid and solid contents 1:1 v/v) were collected before morning feeding, kept in pre-warmed thermo containers at 39°C under anaerobic conditions. About 500 mL of ruminal fluid was collected from all ewes of each treatment. To avoid saliva contamination, the first 50 mL of the rumen fluid samples were discarded. The rumen fluid was mixed for 10 s, squeezed through four layers of cheese cloth, and maintained in a water bath at 39°C under continuous CO₂ flushing until inoculation (19). Three incubation runs were performed in three different weeks. Rumen contents obtained
from the three sheep were combined for each run. Animal use for this trial was approved by the technical committee of the Science, Technology & Innovation Funding Authority (STDF), Egypt (project STDF 33413).

A total mixed ration composed of (per kg DM) 300 g berseem clover, 300 g corn silage, 150 g soybean meal, and 250 g yellow corn was used as a substrate. The nutrient contents of feed ingredients and basal diet are shown in Table 1. The in vitro total gas production (TGP) assay was conducted as described by Theodorou et al. (19) and adapted to the semi-automatic system of Mauricio et al. (20). Ground substrate samples (500 mg of DM) were incubated in 120 mL serum bottles (5 bottles per dose of LAB at each time). LAB (isolated and commercial) was included at 0, 1, 2, and 3 g/kg DM substrate. The isolated bacteria obtained in our laboratory under our conditions according to Mauricio et al. (20) was approved by the technical committee of the Science, Technology & Innovation Funding Authority (STDF), Egypt (project STDF 33413).

During the first week of lactation, thirty lactating Holstein cows (633 ± 25.4 kg body weight, 7 ± 1 parity, 7 ± 1 days in milk, with a previous milk production of 24 ± 2.2 kg/d, were assigned randomly to one of three experimental treatments in a complete randomized design with 10 cows per treatment for 60 days.

Cows were divided into three barns in soil-surfaced free stalls (122 × 175 cm²/cows), under shade, without any bedding and with free access to water. Cows were fed ad libitum a diet containing (per kg DM) 300 g berseem clover, 300 g corn silage, 150 g soybean meal and 250 g grounded corn grain, to meet their nutrient requirements according to NRC (24) recommendations for 650 kg cow with 20 kg DM intake and 35 kg daily milk production. Animals were fed 10% more of the expected dry matter intake to ensure collection of orals. The diet fed to cows was the same for the in vivo experiment.

Cows were fed their diets without any additives (control treatment) or supplemented with 2 g/kg feed daily of E. faecium EGY NRC1 (isolated to contain 1.1 × 10⁹ CFU/g) or commercial E. faecium NCIMB 11181 (isolated to contain 2 × 10¹² CFU/g; ADM Protexin Limited, Lopen Head, Somerset, Table 1: Chemical composition of ingredients and control diet used in the in vitro and in vivo experiments (g/kg of dry matter).

| Ingredient               | Yellow corn² | Soybean meal³ | Corn silage³ | Berseem clover³ | Basal diet³ |
|--------------------------|--------------|---------------|--------------|-----------------|-------------|
| Dry matter, wet weight   | 896          | 898           | 891          | 889             | 893         |
| Organic matter           | 985          | 930           | 915          | 870             | 921         |
| Crude protein            | 83           | 422           | 77           | 170             | 158         |
| Ether extract            | 51           | 48            | 21           | 32              | 36          |
| Ash                      | 15           | 70            | 85           | 130             | 79          |
| Non-structural carbohydrates | 666       | 313           | 286          | 258             | 377         |
| Neutral detergent fiber  | 185          | 147           | 531          | 410             | 351         |
| Acid detergent fiber     | 37           | 65            | 320          | 270             | 196         |

²Analyzed values. ³Calculated values. Used as a diet for all treatments. *Non-structural carbohydrates = 1,000–(neutral detergent fiber + crude protein + ether extract + ash).
TA13 5JH UK). The doses of probiotics were based on the results obtained from Experiment 1 (in vitro experiment). Diets were offered twice a day at 08:00 and 16:00 h. The additives were mixed with all feedstuffs using a feeding wagon. Before use, probiotics were kept at 4°C. Samples of feed ingredients were taken daily, composited weekly, dried at 60°C in a forced-air oven for 48 h (method 930.15) (22) and stored for chemical analyses.

The total mixed ration was prepared and distributed using a horizontal mixer system (DeLaval, Ontario, Canada) after mixing for 20 min. Samples of feed were taken daily, composited weekly, dried at 60°C in a forced-air oven for 48 h (22) and stored for chemical analysis. The nutrient contents of the feed ingredients are shown in Table 1. Cows were weighed on a digital multi-purpose platform scale at the beginning and at the end of the experiment.

Nutrient intake and digestibility
Feed intake was recorded daily by weighing the total daily amount of feed offered to each cow and the total daily amounts of weighed orts. On the 4th and 8th week, nutrient digestibility trials were conducted, in which acid insoluble ash was used as an internal indigestibility marker (25). Acid-insoluble ash contents of feeds and feces were determined gravimetrically after drying, burning, boiling of ash in hydrochloric acid, filtering and washing of the hot hydrolysate, and re-burning. Coefficients of digestion were calculated according to Ferret et al. (26).

For the digestibility trial, fecal samples were collected from all cows twice daily at 09:00 and 16:00 h, dried at 60°C for 48 h in a forced-air oven and pooled by cow. The nutritive value of diets expressed as total digestible nutrients (TDN), digestible energy (DE), metabolizable energy (ME), net energy for lactation (NEL) were calculated according to NRC (24) equation, while the net energy requirements for lactation equivalent of 1 kg of standard air-dry barley (UFL) was calculated according to INRA (27) equation.

The dried feed, orts and fecal samples were ground to pass a 1-mm screen using a Wiley mill and analyzed for DM (method ID 930.15), ash (method ID 942.05), nitrogen (method ID 954.01) and ether extract (EE; method ID 920.39) according to the AOAC (22) official methods. Neutral detergent fiber was determined by the procedure of Van Soest et al. (28) with the use of alpha amylase and sodium sulphite. Acid detergent fiber was analyzed according to the AOAC (22). Lignin was analyzed according to Van Soest et al. (28).

Sampling and analysis of blood serum
On the last day of the 4th and 8th weeks of the experiment, individual blood samples (10 mL) were taken before morning feeding at 08:00 h from the jugular vein. Blood samples were centrifuged at 4,000 ×g for 20 min at 4°C. The serum was separated into 2 mL Eppendorf tubes and frozen at −20°C until analysis. By using specific kits (Spinreact, Ctra. Santa Coloma, Girona, Spain) and following the manufacturers’ instructions, blood serum samples were analyzed for total protein, albumin, globulin, urea-N, glucose, cholesterol, triglycerides, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). The globulin concentration was calculated by subtracting the albumin values from their corresponding total protein values.

Milk sampling, and milk composition
Cows were milked (DeLaval parallel parlor P2100, SE-147 21 Tumba, Sweden) three times daily at 04:00, 12:00 and 20:00 h, and individual milk samples (30 g/kg of milk yield) were collected at each milking. A mixed sample of milk (proportional to amounts isolated in each milking time) was taken daily every 2 weeks to determine milk composition. Milk samples were analyzed using infrared spectrophotometry (Ekomilk-M ultrasonic milk analyzer, EON Trading 2000, INC, Bulgaria).

The gross energy content was calculated according to Tyrrell and Reid (29). The milk energy output (MJ/d) was calculated as milk energy (MJ/kg) × milk yield (kg/d). The energy corrected milk (ECM) and 4% fat corrected milk (FCM) were calculated according to Šjanja et al. (30) and Tyrrell and Reid (29), respectively.

Statistical analyses
Data from in vitro measurements were analyzed using the GLM procedure of SAS (SAS Inst. Inc. Cary, NC, USA) in a completely randomized design using the following model: $Y_{ij} = \mu + D_i + E_{ij}$, where $Y_{ij}$ represents the measured variable, $\mu$ is the overall mean, $D_i$ is the LAB dose, and $E_{ij}$ is the experimental error. Data from each of the three runs within the same sample were averaged prior to the statistical analysis. Polynomial (linear and quadratic) contrasts were used to examine dose responses for increasing levels of LAB.

Data from in vivo measurements were analyzed as a completely randomized design with repeated measures using the PROC MIXED procedure of SAS (SAS Institute, Cary, NC, USA), considering sampling time as repeated measures and individual cow as the experimental unit. Data for variables measured daily for each week were averaged before statistical analyses. The statistical model included the treatment effect, week effect and the treatment × week interaction. Animal nested within treatment was considered the random effect, while treatment was the fixed effect. Two covariance structures were considered in the REPEATED statement in PROC MIXED: compound symmetry (cs) and auto-regressive [AR(1)]. The error structure, with the lowest Akaike information criteria, that fits the statistics was selected for the model. When the F-test was significant at
P < 0.05, means were compared by applying the probability of difference option of the least squares means statement. Significance was declared at P < 0.05.

Results

Experiment 1 (in vitro experiment)

Results of TGP and CH₄ proportions differed between the isolated and commercial strains of E. faecium (Table 2). The inclusion of isolated E. faecium linearly and quadratically increased (P<0.001) TGP, while linearly and quadratically decreased (P < 0.05) proportional CH₄, CH₄ production (per g dDM, g dOM, g dNDF, g dADF) and pH, and increased SCFA (linear and quadratic effects, P < 0.001). Linear increases in dDM and dOM were observed with the isolated E. faecium, with no effect on dNDF or dADF. The inclusion of isolated E. faecium did not affect the concentration of ruminal NH₃-N.

Increased TGP and decreased proportional CH₄ (linear and quadratic effects, P < 0.01) were observed with the inclusion of the commercial E. faecium strain; however, CH₄ production (per g dDM, g dOM, g dNDF, g dADF) and pH linearly decreased (P < 0.01), and dDM, dOM, SCFA and ruminal NH₃-N concentration increased, without affecting dNDF and dADF.

Experiment 2 (lactation experiment)

Feed intake, nutrient digestibility, and blood measurements

Feeding E. faecium diets did not affect total feed intake (Table 3). Compared to the control, the highest (P < 0.01) DM, NDF and ADF degradabilities were observed with the isolated E. faecium followed by the commercial E. faecium, while both isolated and commercial E. faecium improved (P < 0.01) OM, CP, EE and NSC digestibility. The isolated E. faecium followed by the commercial E. faecium showed higher (P < 0.01) diet’s nutritive value calculated as TDN, DE, ME, NEL, and UFL compared to the control treatment. Both the probiotic strains influenced an increased (P < 0.05) intake of digestible OM, TDN, ME and digestible CP compared to control, while between the strains the improvement in TDN, ME intake was superior (P < 0.05) in isolated than the commercial strain.

Isolated or commercial E. faecium did not affect concentrations of serum total protein, albumin, globulin, albumin:globulin ratio and urea-N (Table 4). Both isolated and commercial E. faecium increased serum glucose (P = 0.002) and decreased serum cholesterol (P = 0.002). The commercial E. faecium decreased (P = 0.002) serum triglycerides and ALT (P = 0.038), while the isolated E. faecium decreased serum AST (P = 0.023).

Milk production, milk composition, and milk fatty acids

Compared to the control, the isolated E. faecium followed by the commercial E. faecium increase (P < 0.001) daily milk production (actual, ECM and FCM), daily milk component yields and milk energy output (Table 5). Moreover, the isolated E. faecium followed by the commercial E. faecium improved feed efficiency compared to the control treatment. Treatments did not affect the concentrations of milk components.

Both E. faecium strains decreased the proportion of C23:0 (P = 0.005) and increased (P = 0.017) C18:1 trans-9 in milk (Table 6). Compared to the control treatment, the isolated E. faecium increased (P < 0.05) the proportion of C18:1 trans-9, C18:2 cis-9-12 and C18:2 trans-10 cis-12, while the commercial E. faecium did not affect them.

Discussion

As shown in Figure 1, the MALDI-TOF MS showed that the isolated strain of E. faecium possess antimicrobial activity which was initially identified with a high confidence value of 2.31 that indicated a reliable identification of the isolate up to species level. This result agreed with that of 16S rDNA sequencing data (31). The phylogenetic analysis and the 16S rDNA sequencing assigned all the E. faecium EGY NRC1 isolates belonged to E. faecium.

Experiment 1 (in vitro experiment)

The inclusion of isolated E. faecium (both strains) increased TGP. Generally, production of gases depends mainly on the composition and degradability of the incubated substrate and the concentration of the soluble components in the incubated substrates (32–34). In the present experiment, the composition of the diet and soluble components are the same between treatments indicating that the differences are mainly due to the strains of E. faecium. Jiao et al. (35) stated that specific LAB strains interact with rumen microorganisms to alter rumen fermentation with different modes of action in the rumen.

One promising area of research for the use of LAB in ruminant nutrition is its potential for reducing CH₄ emissions (36). The isolated and commercial E. faecium decreased proportional CH₄ and CH₄ production per unit of degraded DM, OM, NDF and ADF which may be related to the reduced methanogenesis by stimulating the growth of lactate-utilizing bacteria such as Selenomonas ruminantium, Megasphaera elsdenii, and Veillonella parvula, which promotes H₂ and CO₂ sinks during the conversion of lactate to propionate (37). Moreover, LAB stimulates scavenging of hydrogen atoms to form propionate causing a lack of hydrogen as the main substrate for methanogenic bacteria (36).
**TABLE 2**  *In vitro* fermentation from Experiment 1 (mean values), where a basal diet was supplemented with isolated or commercial *E. faecium* as probiotics at 1, 2 or 3 g/kg DM.

|                      | Control | Isolated probiotics<sup>a</sup> | Commercial probiotics<sup>a</sup> | Pooled SEM | Isolated probiotics | Commercial probiotics | Control vs. others | Isolated vs. Commercial |
|----------------------|---------|---------------------------------|----------------------------------|------------|---------------------|----------------------|---------------------|------------------------|
|                      | 1       | 2                               | 3                                | 1          | 2                   | 3                    | Linear             | Quadratic              |                      |
| TGP, mL/g DM         | 114     | 122                             | 121                              | 123        | 115                 | 129                  | 1.1                | <0.001                 | <0.001                | 0.001                |
| CH<sub>4</sub>, %     | 25.1    | 20.4                            | 18.5                             | 19.9       | 20.3                | 21.2                 | 0.36               | <0.001                 | <0.001                | 0.001                |
| CH<sub>4</sub>/g dDM  | 59.7    | 47.9                            | 42.9                             | 45.5       | 47.7                | 42.8                 | 45.0               | 1.26                   | <0.001                 | <0.001                | <0.001                | <0.001                | 0.813                |
| CH<sub>4</sub>/g dOM  | 52.3    | 42.5                            | 38.2                             | 40.4       | 42.6                | 39.9                 | 40.5               | 1.05                   | <0.001                 | <0.001                | <0.001                | <0.001                | 0.473                |
| CH<sub>4</sub>/g dNDF | 79.9    | 62.5                            | 55.0                             | 58.7       | 65.8                | 60.6                 | 61.9               | 3.53                   | <0.001                 | 0.005                 | 0.007                 | 0.036                 | <0.001                | 0.173                |
| CH<sub>4</sub>/g dADF | 112     | 86                              | 74                               | 80         | 91                  | 83                   | 88                | 7.1                    | 0.002                  | 0.032                 | 0.016                 | 0.080                 | 0.008                | 0.244                |
| dDM, g/kg            | 482     | 519                             | 546                              | 532        | 521                 | 569                  | 528               | 13.2                   | 0.006                  | 0.061                 | 0.003                 | 0.004                 | 0.006                | 0.505                |
| dOM, g/kg            | 549     | 585                             | 611                              | 597        | 583                 | 613                  | 586               | 14.0                   | 0.010                  | 0.086                 | 0.032                 | 0.040                 | 0.004                | 0.726                |
| dNDF, g/kg           | 365     | 401                             | 427                              | 413        | 379                 | 407                  | 399               | 20.4                   | 0.072                  | 0.235                 | 0.162                 | 0.591                 | 0.084                | 0.280                |
| dADF, g/kg           | 266     | 292                             | 318                              | 304        | 278                 | 297                  | 290               | 19.5                   | 0.119                  | 0.321                 | 0.308                 | 0.633                 | 0.160                | 0.321                |
| pH                   | 6.65    | 6.38                            | 6.33                             | 6.35       | 6.35                | 6.30                 | 6.33              | 0.038                  | <0.001                 | 0.006                 | <0.001                | <0.001                | <0.001                | 0.377                |
| SCFA, mmol/L         | 1.18    | 1.37                            | 1.45                             | 1.45       | 1.38                | 1.41                 | 1.41              | 0.036                  | <0.001                 | 0.010                 | <0.001                | 0.006                 | <0.001                | 0.412                |
| NH<sub>3</sub>-N, mg/dL | 12.7   | 13.1                            | 13.2                             | 13.1       | 13.0                | 13.2                 | 13.1              | 0.18                   | 0.064                  | 0.199                 | 0.111                 | 0.235                 | 0.031                | 0.710                |

<sup>a</sup>The isolated product contained *E. faecium* EGY_NRC1 registered in the NCBI with accession number MW856655 and contained $1.1 \times 10^9$ CFU/g product evaluated at 1, 2 or 3 g/kg DM.

<sup>b</sup>The isolated product contained *E. faecium* NCIMB 11181 with a total viable count $2 \times 10^{12}$ CFU/g (ADM Protexin Limited, Lopen Head, Somerset, TA13 5JH UK) evaluated at 1, 2 or 3 g/kg DM.
TABLE 3  Experiment 2: Intake, nutrient digestibility and nutritive value of diet supplemented with isolated and commercial E. faecium as probiotics and fed to lactating Holstein cows.

| Diet | Control | Isolated | Commercial | SEM | Diet Control vs. others Isolated vs. Commercial |
|------|---------|----------|------------|-----|--------------------------------------------------|
| Intake, kg/cow/d | 19.0 | 20.1 | 19.5 | 0.43 | 0.184 | 0.127 | 0.300 |
| Digestibility, g absorbed/kg ingested | | | | | | | |
| Dry matter | 586c | 643a | 632b | 2.50 | <0.001 | <0.001 | 0.005 |
| Organic matter (OM) | 627b | 676a | 670a | 3.00 | <0.001 | <0.001 | 0.121 |
| Crude protein (CP) | 603b | 650a | 635a | 5.70 | <0.001 | <0.001 | 0.069 |
| Ether extract | 653b | 702a | 696a | 5.90 | 0.001 | 0.003 | 0.631 |
| Non-structural carbohydrates | 684b | 732a | 723a | 4.30 | <0.001 | <0.001 | 0.147 |
| Neutral detergent fiber | 566c | 637a | 615b | 5.20 | <0.001 | <0.001 | 0.006 |
| Acid detergent fiber | 511c | 582a | 559b | 5.20 | <0.001 | <0.001 | 0.006 |
| Nutritive value | | | | | | | |
| TDN (g/kg DM)b | 612c | 666a | 652b | 2.70 | <0.001 | <0.001 | 0.001 |
| DE (Mcal/kg DM)b | 2.70c | 2.94a | 2.88b | 0.01 | <0.001 | <0.001 | 0.009 |
| ME (%) | 2.73c | 2.97a | 2.91b | 0.01 | <0.001 | <0.001 | 0.002 |
| NEL (Mcal/kg DM)b | 1.38c | 1.51a | 1.48b | 0.007 | <0.001 | <0.001 | 0.004 |
| UFL (Mcal/kg DM)c | 2.43c | 2.66a | 2.60b | 0.01 | <0.001 | <0.001 | 0.007 |
| Digestible OM intake, kg/cow/d | 10.6b | 12.1a | 11.6a | 0.35 | 0.004 | 0.002 | 0.345 |
| TDN intake, kg/cow/d | 11.6c | 13.4a | 12.7b | 0.36 | <0.001 | <0.001 | <0.001 |
| ME intake, Mcal/cow/d | 51.9c | 59.7a | 56.7b | 1.44 | <0.001 | <0.001 | <0.001 |
| Digestible CP intake, kg/cow/d | 1.81b | 2.06a | 1.95a | 0.031 | 0.005 | 0.004 | 0.071 |

Means in the same row with different letters differ (P < 0.05); SEM, standard error of the mean. aDiet: Control diet contained 30% berseem clover + 30% corn silage + 15% soybean meal + 25% yellow corn, without additives, or supplemented with E. faecium EGY_NRC1 (isolated to contains 1.1 × 10^9 CFU/g) or commercial E. faecium NCIMB 11181 (isolated to contains 2 × 10^12 CFU/g) at 2 g/kg feed daily/cow. bTDN, total digestible nutrients; DE, Digestible energy; ME, Met abolizable energy; NEL, Net energy for lactation. All have been calculated according to NRC equation. cUFL = unité fourragère du lait (net energy requirements for lactation equivalent of 1 kg of standard air-dry barley) calculated according to INRA equation.

TABLE 4  Experiment 2: Blood parameters of Holstein cows fed diets supplemented with isolated or commercial E. faecium as probiotics.

| Diet | Control | Isolated | Commercial | SEM | Diet Control vs. others Isolated vs. Commercial |
|------|---------|----------|------------|-----|--------------------------------------------------|
| Total proteins, g/dL | 9.58 | 9.93 | 9.96 | 0.15 | 0.193 | 0.073 | 0.897 |
| Albumin, g/dL | 5.35 | 5.60 | 5.52 | 0.14 | 0.480 | 0.252 | 0.715 |
| Globulin, g/dL | 4.23 | 4.33 | 4.43 | 0.13 | 0.589 | 0.385 | 0.590 |
| Albumin:globulin ratio | 1.29 | 1.32 | 1.27 | 0.06 | 0.833 | 0.943 | 0.553 |
| Urea-N, mg/dL | 78.9 | 76.8 | 81.3 | 1.54 | 0.144 | 0.917 | 0.052 |
| Glucose, mg/dL | 74.0b | 81.5a | 80.3a | 1.39 | 0.002 | 0.004 | 0.533 |
| Cholesterol, mg/dL | 172a | 152b | 144b | 5.20 | 0.002 | 0.007 | 0.313 |
| Triglycerides, mg/dL | 109a | 101a | 97b | 1.30 | 0.002 | 0.002 | 0.034 |
| AST, Units/L | 35.3a | 30.0b | 33.0a | 1.27 | 0.023 | 0.021 | 0.012 |
| ALT, Units/L | 23.5a | 22.3a | 20.6b | 0.82 | 0.038 | 0.049 | 0.016 |

AST, Aspartate aminotransferase; ALT, Alanine aminotransferase. Means in the same row with different letters differ (P < 0.05); SEM, standard error of the mean. aDiet: Control diet contained 30% berseem clover + 30% corn silage + 15% soybean meal + 25% yellow corn, without additives, or supplemented with E. faecium EGY_NRC1 (isolated to contains 1.1 × 10^9 CFU/g) or commercial E. faecium NCIMB 11181 (isolated to contains 2 × 10^12 CFU/g) at 2 g/kg feed daily/cow.

et al. (11) observed a lowered in vitro CH₄ production with LAB supplementation to a silage-based diet prepared with whole crop rice. To confirm our findings, further studies should consider analyzing rumen microbiome. The isolated strain of E. faecium increased in vitro TGP and decreased CH₄ production compared to the commercial strain, with no clear reason indicating the need for experiments on genome sequence and their ability to produce bacteriocins.
and non-ribosomal synthesized peptides for explaining such effects (36). Such possible differences between strains or their metabolites will produce different abilities to shift rumen fermentation patterns, and to inhibit specific rumen bacteria that produce H₂ or methyl-containing compounds that are the substrates for methanogenesis (36). Increasing TGP is not always advantageous but concurrent reduction in CH₄ is definitely advantageous (38). The improved fiber digestion is the most probable reason for the lowered CH₄ production (39).

The isolated and commercial strains increased dDM and dOM without affecting dNDF or dADF due to the high fermentative activities of LAB-probiotics. LAB can enhance the whole digestive process, the metabolic utilization of nutrients, and improve the feed efficiency by producing digestive enzymes (e.g., amylases, chitinases, lipases, phytases, proteases) or by just generating volatile fatty acids and B-vitamins: riboflavin, biotin, B₁₂ vitamin (40). Cao et al. (11) observed increased in vitro DM degradability with LAB administration to total mixed ration silage containing whole crop rice.

The commercial E. faecium increased ruminal NH₃-N concentration; however, the observed concentrations were greater than the optimum level (8.5 to over 30 mg NH₃-N/dL) for rumen microbial proliferation (41). Basso et al. (10) observed no effects on ruminal pH when lambs were fed a diet treated with LAB. However, the isolated and commercial E. faecium decreased fermentation pH, which was somehow mirrored by the obtained SCFA. The isolated and commercial E. faecium increased SCFA concentrations, and this may be related to the improved DM and OM digestibility. So et al. (13) reported increased total SCFA in cows fed diets supplemented with LAB. The observed fermentation pH values in all treatments were greater than the optimum level (5.6) for ruminal fiber degrading and microbial growth (42), without changing ruminal fibrolytic and amylolytic microbial communities (43).

The quadratic effects of treatments (levels of E. faecium) on some parameters are important to emphasize the importance of defining the optimal dose of E. faecium that may improve animal performance. Therefore, the medium dose of E. faecium (2 g/kg feed) was recommended for the in vivo experiment on lactating cows, as this dose showed better effects compared to the low and high doses.
### TABLE 6  Experiment 2: Milk fatty acid profile (g/100 g fatty acids) of lactating Holstein cows fed diets supplemented with isolated or commercial *E. faecium* as probiotics.

| Diet³ | Control | Isolated | Commercial | SEM | Diet | Control vs. others | Isolated vs. Commercial |
|-------|---------|----------|------------|-----|------|-------------------|------------------------|
| C4:0  | 0.81    | 0.81     | 0.75       | 0.017 | 0.138 | 0.247             | 0.089                  |
| C6:0  | 0.97    | 0.95     | 0.99       | 0.016 | 0.391 | 0.876             | 0.206                  |
| C8:0  | 0.93    | 0.90     | 0.93       | 0.017 | 0.400 | 0.587             | 0.238                  |
| C10:0 | 2.68    | 2.74     | 2.75       | 0.128 | 0.907 | 0.686             | 0.960                  |
| C11:0 | 0.20    | 0.20     | 0.19       | 0.010 | 0.866 | 0.942             | 0.625                  |
| C12:0 | 3.33    | 3.38     | 3.35       | 0.193 | 0.983 | 0.892             | 0.920                  |
| C13:0 | 0.46    | 0.46     | 0.47       | 0.015 | 0.895 | 0.705             | 0.826                  |
| C14:0 | 12.3    | 12.2     | 12.3       | 0.180 | 0.829 | 0.709             | 0.664                  |
| C15:0 | 1.07    | 1.10     | 1.08       | 0.015 | 0.524 | 0.466             | 0.408                  |
| C16:0 | 34.4    | 34.2     | 33.9       | 0.240 | 0.502 | 0.343             | 0.536                  |
| C17:0 | 0.66    | 0.63     | 0.65       | 0.078 | 0.974 | 0.866             | 0.900                  |
| C18:0 | 9.36    | 9.20     | 9.21       | 0.205 | 0.817 | 0.559             | 0.974                  |
| C20:0 | 0.12    | 0.12     | 0.12       | 0.010 | 0.928 | 0.721             | 1.000                  |
| C22:0 | 0.05    | 0.05     | 0.05       | 0.082 | 0.936 | 0.737             | 1.000                  |
| C23:0 | 0.04a   | 0.03b    | 0.03b      | 0.002 | 0.005 | 0.023             | 0.610                  |
| C24:0 | 0.04    | 0.04     | 0.04       | 0.009 | 0.494 | 0.550             | 0.329                  |
| ∑ saturated fatty acids (SFA) | 67.4 | 66.9 | 66.8 | 0.480 | 0.698 | 0.531 | 0.868 |
| C14:1 cis-9 | 0.28 | 0.28 | 0.27 | 0.009 | 0.740 | 0.651 | 0.711 |
| C14:1 trans-9 | 0.99 | 0.98 | 0.99 | 0.004 | 0.650 | 0.885 | 0.450 |
| C16:1 cis-9 | 0.34 | 0.32 | 0.34 | 0.026 | 0.859 | 0.466 | 0.624 |
| C16:1 trans-9 | 1.86 | 1.83 | 1.81 | 0.037 | 0.668 | 0.227 | 0.663 |
| C18:1 cis-9 | 19.9 | 20.8 | 21.1 | 0.570 | 0.412 | 0.410 | 0.744 |
| C18:1 trans-9 | 0.24b | 0.26a | 0.26a | 0.007 | 0.017 | 0.037 | 1.000 |
| C18:1 trans 11 | 0.97b | 1.04a | 1.00b | 0.019 | 0.025 | 0.041 | 0.022 |
| ∑ monounsaturated fatty acids | 24.5 | 25.5 | 25.7 | 0.570 | 0.405 | 0.505 | 0.781 |
| C18:2 trans-9,12 | 1.76 | 1.76 | 1.72 | 0.084 | 0.911 | 0.938 | 0.730 |
| C18:2 cis-9,12 | 0.17b | 0.19a | 0.18b | 0.005 | 0.040 | 0.037 | 0.033 |
| C18:2 cis-9,11 trans-11 | 0.40 | 0.41 | 0.40 | 0.024 | 0.986 | 0.405 | 0.892 |
| C18:2 trans-10 cis-12 | 0.01b | 0.02a | 0.01b | 0.002 | 0.029 | 0.036 | 0.033 |
| C18:3 cis-9,12,15 | 0.11 | 0.10 | 0.10 | 0.005 | 0.441 | 0.669 | 0.346 |
| C18: 3 cis-9,12,15 | 0.40 | 0.39 | 0.40 | 0.026 | 0.954 | 0.829 | 0.806 |
| C20:3 cis-8,11,14 | 0.09 | 0.09 | 0.09 | 0.002 | 0.364 | 1.000 | 0.201 |
| C20:4 cis-5,8,11,14 | 0.12 | 0.13 | 0.12 | 0.009 | 0.898 | 0.231 | 0.711 |
| C20:5 cis-5,8,11,14,17 | 0.03 | 0.03 | 0.03 | 0.006 | 0.604 | 0.442 | 0.353 |
| C22:5, cis-7,10,13,16,19 | 0.17 | 0.16 | 0.16 | 0.004 | 0.354 | 0.265 | 0.450 |
| ∑ polyunsaturated fatty acids | 3.25 | 3.26 | 3.20 | 0.075 | 0.818 | 0.220 | 0.587 |
| ∑ unsaturated fatty acids (UFA) | 27.8 | 28.8 | 28.9 | 0.630 | 0.478 | 0.821 | 0.853 |
| ∑ conjugated linoleic acid | 0.41 | 0.42 | 0.41 | 0.024 | 0.969 | 0.882 | 0.860 |
| UFA: SFA | 0.41 | 0.43 | 0.43 | 0.012 | 0.493 | 0.276 | 0.848 |

Means in the same row with different letters differ (P < 0.05); SEM, standard error of the mean. *Diet: Control diet contained 30% berseem clover + 30% corn silage + 15% soybean meal + 25% yellow corn, without additives, or supplemented with *E. faecium EGY_NRC1* (isolated to contains 1.1 × 10⁹ CFU/g) or commercial *E. faecium* NCIMB 11181 (isolated to contains 2 × 10¹² CFU/g) at 2 g/kg feed daily/cow. ³C18:2 cis-9 trans-11 + C18:2 trans-10 cis-12.
Experiment 2 (lactation experiment)

It was not possible to obtain ruminal contents from cows because the experiment was done in a commercial farm without access to rumen-fistulated lactating cows. Therefore, the in vitro approach shows results that may partially explain and/or support the outcomes of the lactation experiment.

Feed intake, nutrient digestibility, and blood measurements

_E. faecium_ supplementation did not affect feed intake which partly indicates unchanged feed palatability or acceptability. Other studies reported minor effects on feed intake in lambs and ewes fed with probiotics (8, 44), while So et al. (13) observed increased feed intake with LAB supplementation to lactating cows.

The isolated _E. faecium_ improved the digestibility of DM, NDF and ADF compared to the commercial _E. faecium_; however, both isolated and commercial strains improved OM, CP, EE and NSC digestibility revealing the potential of _E. faecium_ for improving nutrient digestibility. Similarly, So et al. (13) observed improved nutrient digestibility with LAB supplementation in lactating cows. Fiber degradability results are not consistent with those of the in vitro experiment (Experiment 1), which may be due to the conditions of both experiments (in vitro vs. in vivo conditions) and the fact that feeding bacterial direct fed microbial to livestock is based primarily on potential postruminal effects which is not available in the in vitro experiments (35). In this regard, probiotics change rumen fermentation rates and patterns (45), with beneficial effects on the gastrointestinal tract and rumen (46). Additionally, the supplement contains LAB which has a strong inhibitory effect on gastrointestinal infection by pathogens _via_ the production of antimicrobial agents (46). It is expected that _E. faecium_, especially, the isolated strain, improved growth or activity of ruminal cellulolytic microbial populations and stabilizes the rumen pH (47), leading to improved nutrient digestion (45) and synthesis of microbial proteins (48). As previously noted, the isolated strain improved digestibility of DM, NDF, and ADF, and diet's nutritive value compared to the commercial strain, which confirm our assumption that the genome of both strains differs. The possible differed production of metabolites and bacteriocins may affect the composition of rumen microbiome, especially in those involved in fiber digestion (36). As observed in this study, previous experiments on lambs (49, 50), and lactating ewes (8) reported that probiotics improved nutrient digestibility.

All the measured serum biochemical parameters were within the standard physiological ranges for healthy cows (51). Treatments did not affect the concentrations of serum total protein, albumin, globulin, albumin: globulin ratio and urea-N indicating minimal effects on cow's nutritional status, muscle protein catabolism and kidney function (52). Both _E. faecium_ strains increased serum glucose because of improved apparent OM and NSC. The levels of serum glucose were above the range (40–60 mg/dL), indicating an adequate energy supply for cows without risk of negative energy balance occurring (53). Further studies should follow on these findings as _E. faecium_ supplementation could be helpful during the transition period.

Both _E. faecium_ strains decreased serum cholesterol while commercial _E. faecium_ decrease serum triglycerides showing the ability of _E. faecium_ bacteria to deconjugate bile salts by a specific hydrolase causing a reduction in cholesterol and triglycerides absorption at the intestinal level (54). Additionally, the commercial _E. faecium_ decreased serum ALT, while isolated _E. faecium_ decreased serum AST showing its potential to improve liver activity, function, and health in cows (55).

Milk production, milk composition, and milk fatty acids

In this study, both isolated and commercial _E. faecium_ increased daily milk production by 17.1 and 11.7%, ECM by 21.4 and 14.8% and FCM 20.9 and 14.2%, respectively which is similar to other studies (8, 13, 44, 56) that reported a positive relationship between supplementation of ruminant diets with probiotics and animal performance. The improved nutrient digestibility and increased blood glucose with probiotics supplementation can be considered the main reasons for increasing milk production (44). An improvement in digestibility and intake of nutrients (ME and TDN) supported release of important nutrients required for milk components synthesis (41). Moreover, the improved feed efficiency with the additives is another probable reason for the improved performance (41). As previously noted in Experiment 1, _E. faecium_ decreased CH4 production indicating that a possible suppression in CH4 production would have redistributed energy for improved milk production (57). In this study, the antagonism of pathogenic organisms _via_ antimicrobial effects, competition for adhesion sites or nutrients, stimulation of host defines mechanisms and inhibition of bacterial toxins can partially explain the improved milk production (9, 45).

Moreover, LAB increases the release of different endogenous substances, including antibacterial substances, nutrients, antioxidants, growth factors and coagulating agents, enhances performance and reduces the incidence of diarrhea by increasing the number of beneficial microorganisms in the rumen (45, 58) and enhancing animal health (59), which could also explain the increased milk production. As a result of unchanged feed intake and increased daily milk production, the isolated and commercial _E. faecium_ improved feed efficiency by 11.2 and 8.7% (milk: intake ratio), 14.5 and 11.8 (ECM: intake ratio), and 14 and 11.3% (FCM: intake ratio), respectively. Frizzo et al. (60) observed that supplementing diets of lactating cows with LAB including _E. faecium_ improved feed efficiency.
The weak effect of treatment on the concentrations of milk components is inconsistent with other experiments (8) that reported some changes in the concentrations of total n-3, n-6 fatty acids, polyunsaturated fatty acids and conjugated linoleic acids components when sheep were fed diet supplemented with LAB. However, more experiments are required to explore these effects.

Plasma uptake of fatty acids is responsible for about half of milk fatty acids and the rest amount is synthesized in the mammary gland (61, 62). The improved fiber digestion with the supplementation might be associated with altered milk fatty acid profiles. Both E. faecium strains increased the proportion of C18:1 trans-9, while the isolated E. faecium increased the proportion of C18:1 trans-9, C18:2 cis-9-12 and C18:2 trans-10 cis-12. The observed changes in milk fatty acids are a result of biohydrogenation of dietary PUFA (63). Further attention should be paid to the use of E. faecium on cow diets as they may increase the formation of bioactive fatty acids such as C18:1 trans-11 and C18:2 cis-9-12.

Conclusion

Daily supplementation of cows with E. faecium (isolated and commercial) at 2 g/kg DM feed improved in vitro nutrient degradation and cows feed digestion, milk production and feed efficiency. The isolated strain of E. faecium showed better results compared to the commercial strain. Minimal effects were observed with E. faecium supplementation on milk fatty acid profile. Our data could be useful for producers looking for probiotics generated from byproducts for improving feed utilization and milk production.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The names of the repository/repositories and accession number(s) can be found below: NCBI accession number MW856655.

Ethics statement

The animal study was reviewed and approved by the Technical Committee of the Science, Technology & Innovation Funding Authority (STDF), Egypt (project STDF 33413).

Author contributions

HA, AK, and HM: conceptualization, methodology, validation, formal analysis, visualization, and supervision. AK and EV-B-P: writing—original draft preparation and writing—review and editing. HA and AK: investigation and data curation. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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