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Research article

Effect of replacement of berseem clover by almond hulls from Palestine or Tunisia with *Pichia manshurica* yeast as feed additives in mixed diet on rumen fermentation, methane emissions, and protozoa counts, in vitro

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Abstract: This study was conducted to evaluate the effects of combined supplementation of two rumen-isolated *Pichia manshurica* yeast strains (FFNLYFC1and FFNLYFC2), which were combined 1:1 and dosed at 1.4×10^8 CFU/g dry matter (DM) from each strain on *in vitro* ruminal fermentation

of diets in which almond hulls from Palestine (AHP) or Tunisia (AHT) replaced berseem clover (*Trifolium alexandrinum*) as the primary forage source. Five experimental treatments were evaluated at a 60:40 forage-to-concentrate ratio: a control diet (berseem clover hay); AHP without yeast (AHP–); AHP with yeast (AHP+); AHT without yeast (AHT–); and AHT with yeast (AHT+). Three independent biological runs were performed using rumen fluid from three water buffalo males. The asymptotic gas production potential (*b*) was highest in the control and AHP+ (149.3 and 149.2 mL/g DM, respectively; P = 0.016) and significantly lower in AHT diets (140.4–141.1 mL/g DM). True dry matter digestibility was highest in AHP+ (81.4%) and lowest in AHT– (77.9%). The AHP+ diet yielded the highest total volatile fatty acid (VFA) concentration (129.6 mmol/L), representing a 37.6% increase over the control (94.2 mmol/L), alongside the greatest propionate proportion (35.4% vs. 21.1% in the control; P = 0.001). Microbial biomass production was highest in AHP+ (523.3 mg/g DM; P = 0.003). True digestible organic matter was significantly greater in AHT– and AHT+ (812.9 and 813.8 g/kg DM, respectively) than in the control (786.4 g/kg DM), reflecting lower lignification in Tunisian hulls. The true neutral detergent fiber degradability (TNDF) was markedly higher in the control (539.1 g/kg DM NDF than in all almond hull diets (357.4–421.5 g/kg NDF; P < 0.001), though yeast supplementation improved TNDF in AHP diets by 17.9%. Methane production per gram of DM was highest in AHP+ (32.6 mL/g DM; P = 0.039), though methane (CH_4) per unit of truly digested organic matter did not differ significantly among treatments (P = 0.059), indicating that the elevated absolute CH_4 in AHP+ reflects greater total fermentation activity rather than enhanced methanogenesis per se. Ruminal pH and protozoal counts were not significantly different among treatments. These findings indicated that *P. manshurica* yeast supplementation substantially enhanced fermentation efficiency and VFA output in Palestinian almond hull diets and exerted a meaningful but more modest benefit in Tunisian hull diets, with the geographic origin of the almond hulls appearing to modulate the fermentation response to yeast inclusion. *P. manshurica*-supplemented Palestinian almond hull diets (AHP+) produced the best combined fermentation outcome among the almond hull treatments evaluated.

Keywords: *P. manshurica*; almond hulls; in vitro rumen fermentation; volatile fatty acids; methane

1. Introduction

Sustainable livestock production in arid and semi-arid Mediterranean regions increasingly depends on the identification and valorization of locally available, non-conventional feedstuffs that can partially or fully replace conventional forages without compromising animal productivity or environmental performance. In Tunisia and Palestine, where almond (*Prunus dulcis*) cultivation is widespread, almond hulls (AH) represent an abundant agro-industrial residue generated annually during almond processing. These hulls accumulate as a fibrous co-product that must be managed responsibly, yet they remain underutilized in ruminant feeding systems [1]. The nutritional characteristics of almond hulls (moderate fiber content, low crude protein relative to leguminous forages, and variable lignification depending on cultivar and geographic origin) make them a candidate roughage source for ruminant diets, provided that constraints to their ruminal fermentability are adequately addressed [2,3].

Almond hulls from Palestine and Tunisia exhibit distinct chemical profiles, particularly in neutral

detergent fiber (NDF), acid detergent lignin (ADL), and hemicellulose contents, as demonstrated by Zoabi et al. [1], who compared the *in vitro* ruminal fermentation of almond hulls from both origins using chemical treatments, including NaOH, urea, and polyethylene glycol. That study established that almond hulls from Palestine had lower fiber contents than those from Tunisia, and that chemical processing differentially affected fermentation parameters depending on geographic origin. Notably, the almond hull batches evaluated in this study showed the reverse pattern, with the Palestinian hulls exhibiting higher NDF, ADL, and hemicellulose content than the Tunisian hulls; this likely reflects the well-documented lot-to-lot and cultivar variability in almond hull composition and underscores the need for batch-specific chemical characterization when interpreting fermentation outcomes. These findings highlight the importance of origin-specific nutritional evaluation when designing supplementation strategies for almond hull-based ruminant diets [4,5].

Microbial feed additives, particularly direct-fed microbials (DFM) based on yeast, have been extensively studied for their capacity to modulate rumen fermentation [6]. Yeast culture consists primarily of extracellular metabolites, residual culture medium components, cell wall fragments, and viable yeast cells; in this study, by contrast, we used live, viable *Pichia manshurica* cells dosed at a defined colony-forming-unit (CFU) concentration, rather than a commercial yeast culture product [7]. Supplementation with yeast has been reported to stimulate the growth of cellulolytic and hemicellulolytic bacteria by scavenging residual oxygen, to stabilize ruminal pH by supporting lactate-consuming bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium*, and to shift the volatile fatty acid (VFA) profile toward a more propionate-rich pattern conducive to efficient gluconeogenesis [8,9]. This propionate-rich shift, driven largely by yeast-mediated stimulation of lactate-utilizing and succinate/propionate-producing bacteria, is well documented for several yeast species [8,9]. Amin and Mao [10] reported that yeast supplementation can increase total VFA concentration and contribute to ruminal pH stabilization, primarily through support of lactate-utilizing bacteria, thereby reducing the risk of subacute ruminal acidosis under certain feeding conditions; however, the magnitude and consistency of these responses vary with basal diet, yeast dose and viability, and feeding strategy, a consideration particularly relevant when high-fermentability by-product feeds are substituted for conventional forages [11,12]. Furthermore, yeast-derived metabolic byproducts, including B vitamins, amino acids, and nucleotide precursors, provide growth factors that selectively benefit strict anaerobes, particularly cellulolytic bacteria of the genera *Ruminococcus* and *Fibrobacter*, which lack biosynthetic capacity for many of these compounds [9]. The practical outcome of these multi-mechanism effects is typically an expansion of the fermentable substrate pool accessible to the rumen microbial community, increased microbial biomass production, and improved overall feed conversion, benefits that are especially relevant when the forage source has inherently moderate fermentability, as is the case for almond hulls.

While *Saccharomyces cerevisiae* has dominated yeast supplementation research, interest has shifted toward non-*Saccharomyces* yeast species native to the rumen environment, which may offer greater ecological compatibility and substrate-specific fermentation-modulating activity [13,14]. This potential advantage remains a working hypothesis rather than an established finding and is part of the rationale motivating the present investigation of a rumen-native *P. manshurica* strain. Soltan et al. [13] isolated two strains of *P. manshurica* (FFNLYFC1, GenBank OP600624; FFNLYFC2, GenBank OP600623) from Zaraibi goats rumen contents and demonstrated that their combined supplementation (1:1 ratio) reduced methane (CH_4) by 23.5% and improved ruminal fermentation parameters in aflatoxin B1-contaminated diets. The potential of these strains to enhance the fermentability of fibrous

agro-industrial by-product diets, such as almond hull-based rations, has not been investigated. Understanding this interaction is practically important because the chemical composition of almond hulls, notably their fiber quality and polyphenolic content, is expected to modulate the response to yeast supplementation in ways that cannot be extrapolated from studies on conventional forages or high-concentrate diets.

High-fermentability diets, particularly those combining substantial rapidly fermentable carbohydrate with insufficient effective fiber, may predispose animals to rapid acid accumulation in the rumen, compromising pH stability and fiber-degrading microbial populations [12]. Yeast supplementation under such conditions may serve not only as a fermentation enhancer but also as a pH stabilizer, extending the utility of fibrous by-products in intensive and semi-intensive ruminant systems [8,10]. However, the degree to which *P. manshurica* mediates such effects in almond hull diets of varying geographic origin remains unknown. Therefore, this study was conducted to evaluate the effects of combined *P. manshurica* yeast supplementation on in vitro ruminal fermentation, VFA profiles, nutrient digestibility, microbial biomass production (MBP), metabolizable energy (ME), and CH_4 emissions in ruminant-type diets in which almond hulls from Palestine and Tunisia replaced berseem clover as the forage source. Because rumen fluid was sourced from water buffalo, a large ruminant, the fermentation responses characterized here are discussed in relation to ruminants broadly. The use of rumen fluid from a donor species other than the target species is an established approach in in vitro gas production studies, and inoculum source effects on fermentation outcomes have been characterized [15]. Our objectives were to: (1) compare gas production kinetics and true dry matter digestibility (TDMD) across dietary treatments; (2) quantify VFA concentration and molar proportions; (3) assess true digestible organic matter (TDOM), true neutral detergent fiber degradability (TNDF), MBP, and ME; and (4) evaluate the effects on enteric CH_4 expressed per unit of DM, per unit of TDOM, and per unit of TNDF.

2. Materials and methods

2.1. Experimental location

All experimental incubations were conducted at the Nanotechnology and Greenhouse Gases Laboratory, Faculty of Agriculture, Alexandria University, Egypt. Rumen fluid was obtained from animals slaughtered at the research abattoir of the same institution. No live animal experiments were performed, and all procedures complied with the institutional guidelines for the humane handling of research animals.

2.2. Almond hull collection and preparation

Almond hulls from Palestine (AHP) were collected during August and September 2022 from multiple locations in the Jenin Governorate in collaboration with private agricultural companies. The hulls represented a composite of several locally prevalent almond varieties. After collection, the hulls were sun-dried to reduce moisture content and stored in polyethylene bags at ambient temperature until use. Almond hulls from Tunisia (AHT) were obtained through a commercial supplier and represented varieties grown in the northwestern almond-producing regions of Tunisia. Both hull types were ground to pass a 1-mm screen before chemical analysis and incorporation into experimental diets. Chemical

composition of both hull types and of all experimental diets was determined according to AOAC [16], and fiber fractions were measured following Van Soest et al. [17] using heat-stable alpha-amylase and sodium sulfite.

2.3. Yeast preparation

Two strains of *P. manshurica* (designated FFNLYFC1 and FFNLYFC2) were originally isolated from the ruminal contents of Zaraibi goats at Alexandria University and deposited in GenBank under accession numbers OP600624 and OP600623, respectively [13]. Stock cultures were maintained on yeast extract-peptone-dextrose agar and propagated in liquid medium to achieve the target concentration. Both strains were combined at a 1:1 volumetric ratio, and the combined suspension was added directly to individual fermentation bottles at 1.4×10^8 CFU per gram of DM from each strain. This dose was selected based on prior dose-response experiments in which it produced the most consistent improvements in fermentation parameters without evidence of substrate competition [13].

2.4. Experimental diets and treatments

Five experimental treatments were formulated and incubated in vitro at a 60:40 forage-to-concentrate ratio on a DM basis. The diets were formulated to meet maintenance-level nutrient density guidelines as a general framework for basal nutrient adequacy in the in vitro system. The concentrate component was held constant across all treatments and consisted of ground maize (80 g/kg DM), wheat bran (76 g/kg DM), soybean meal (24 g/kg DM), barley (170 g/kg DM), broken rice (36 g/kg DM), and other ingredients formulated using established small ruminant nutrient density recommendations [18]. One main objective of the study was to evaluate the effects of replacing the forage source, specifically berseem clover with almond hulls, on the chemical composition and nutritional characteristics of the resulting diets. Therefore, berseem clover was replaced with almond hulls on an equal DM basis, while the concentrate component was kept unchanged among treatments. This design enabled the effects associated with changing the forage source to be evaluated without simultaneously altering the composition or inclusion level of the concentrate. Because the almond hulls directly replaced berseem clover on a DM basis rather than being formulated to equalize crude protein, the resulting difference in dietary crude protein between the control and almond hull diets was an inherent consequence of the forage substitution. The control diet contained berseem clover (*Trifolium alexandrinum*) hay as the sole forage. Treatments AHP– and AHP+ contained Palestinian almond hulls without and with *P. manshurica* yeast supplementation, respectively. Treatments AHT– and AHT+ contained Tunisian almond hulls without and with yeast supplementation, respectively. The complete ingredient composition and chemical analyses of all experimental diets are presented in Table 1.

Table 1. Ingredients and chemical composition of the ingredients and experimental diets.

Item	Berseem hay	AHP	AHT	Control	AHP	AHT
Ingredients (g/kg DM)						
Trifolium alexandrinum clover hay				600	0	0
Almond hulls – Palestine				0	600	0
Almond hulls – Tunisia				0	0	600
Ground maize				80	80	80
Wheat bran				76	76	76
Soybean meal				24	24	24
Barley				170	170	170
Broken rice				36	36	36
Calcium carbonate				6	6	6
Sodium chloride				4	4	4
Vitamins and minerals premix				4	4	4
Chemical composition (% DM)						
Organic matter	89.2	91.27	91.82	99.12	99.11	99.54
Crude protein	16.2	5.49	5.65	16.24	9.03	8.88
Neutral detergent fiber	41.7	30.00	33.72	41.72	31.23	28.79
Acid detergent fiber	19.7	11.71	17.73	19.74	11.05	10.90
Acid detergent lignin	3.8	8.49	9.75	3.82	3.32	2.38
Cellulose	15.9	3.22	7.98	15.92	7.73	8.52
Hemicelluloses	22.0	18.29	15.99	21.98	20.18	17.89
Ether extract	2.5	2.60	2.43	2.52	1.24	1.58

AHP: almond hulls from Palestine; AHT: almond hulls from Tunisia. The Almond hulls–Palestine and Almond hulls–Tunisia columns report the chemical composition of the raw, unprocessed hull material prior to inclusion in the diet. Control, AHP, and AHT report the composition of the complete 60:40 forage-to-concentrate mixed diets. The chemical composition of the raw almond hulls prior to inclusion in the diet was previously reported in our companion study [1].

2.5. Rumen fluid collection and in vitro incubation

Rumen fluid was collected from three crossbred water buffalo males (mean body weight 430 ± 5 kg SE) immediately after slaughter. The donor animals had been fed ad libitum *Trifolium alexandrinum* hay and rice straw, supplemented with a commercial concentrate mixture containing 12% crude protein. Contents were transferred into pre-warmed insulated containers at 39 °C under continuous flushing with carbon dioxide (CO_2), homogenized for 10 seconds, filtered through three layers of cheesecloth, and maintained at 39 °C in a water bath under continuous CO_2 flushing until use. The use of buffalo rumen contents was intended to provide a consistent source of active ruminal microorganisms for comparative evaluation of the fermentation characteristics of the experimental diets. This approach has been used for in vitro ruminal fermentation studies, including the use of buffalo-derived rumen contents as inoculum [13]. Although the experimental diets were formulated within a small-ruminant nutritional framework, the inoculum was not species-matched to sheep or goats. Therefore, the buffalo-derived rumen fluid was used to assess relative differences among dietary treatments rather than to directly reproduce the ruminal environment of ruminants. The buffer medium was prepared according

to Menke and Steingass [19]. For each treatment, 500 mg of substrate (1-mm screen) was weighed into ANKOM F57 filter bags, heat-sealed, and placed in 250-mL serum bottles. Each bottle received 30 mL buffer and 15 mL rumen fluid under continuous CO_2 flushing, which were then crimp-sealed and transferred to a forced-air incubator at 39 °C for 24 h [15]. Three blanks (inoculum without substrate) were included per run. Three independent biological runs were conducted with six bottles per treatment per run (18 observations per treatment in total). The incubation system followed Bueno et al. [15], with modifications by Soltan et al. [13].

2.6. Gas production and methane measurement

Headspace pressure was recorded at 4, 8, 12, and 24 h using a digital pressure transducer (PSI-V2, SmartLab, Cairo, Egypt). Gas volume was calculated as: GP (mL) = 6.5465 × p − 0.9573, where p is gas gauge pressure [13]. Net gas production was obtained by subtracting blank values. Cumulative gas production data were fitted to the nonlinear model of France et al. [20]: $y = b \times [1 - e^{-c\,(t-Lag)}]$, where y is gas volume (mL/g DM) at time t (h); *b* is the asymptotic potential gas production from the insoluble but fermentable fraction (*b*, mL/g DM); *c* is the fractional rate constant (/h); and Lag is the discrete lag time (h). Gas yield at 24 h (GY_{24}; mL/g truly degraded DM) and ME (MJ/kg DM) were estimated according to Menke et al. [21]. The partitioning factor (PF; mg truly degraded DM/mL gas) was calculated according to Blümmel et al. [22]. At the end of incubation, 5 mL of headspace gas was withdrawn with a gas-tight syringe, and CH_4 concentration was measured with a laser detector (Gas-Pro-IR, Crowcon, UK) [23]. Methane production was expressed as mL/g DM incubated (CH_4/DM), mL/g TDOM (CH_4/TDOM), and mL/g truly digested NDF (CH_4/TNDF).

2.7. Nutrient digestibility and fermentation parameters

After gas sampling, fermentation was terminated on ice for 5 min. Filter bags were removed, rinsed with cold water until the runoff was clear, and dried at 55 °C for 48 h. TDMD was calculated from the difference between initial and residual DM [22]. For TDOM, dried residues were ashed at 550 °C for 4 h. TNDF was determined on residues according to Van Soest et al. [17]. MBP was estimated from truly degraded DM minus gas volume as described by Blümmel et al. [22]. Ruminal pH was measured immediately upon bottle opening with a calibrated digital pH meter. Protozoal counts were determined by preserving 2 mL of fermentation fluid in methyl-green-formalin-saline (1:1 v/v) and enumerated using a Neubauer hemocytometer at 10× magnification [24], expressed as 10^5 cells/mL. VFA concentration was determined by HPLC (Agilent 1260 Infinity II) following De Baere et al. [25]. A 1-mL aliquot was acidified with 0.2 mL metaphosphoric acid (250 g/L), held for 30 min, centrifuged at 10,000 × g, and filtered through a 0.45-μm membrane. Chromatographic separation used an Eclipse XDB-C18 column with UV detection at 210 nm. Individual VFA concentrations were quantified with a commercial standard mixture (Sigma-Aldrich, Germany), and total VFA (mmol/L) and molar proportions (% of total VFA) were calculated.

2.8. Statistical analysis

Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) in a randomized complete block design and run was the blocking factor: $Y_{ijk} = \mu + T_i + R_j + \varepsilon_{ijk}$,

where Y_{ijk} is the observation, μ is the overall mean, T_i is the fixed effect of dietary treatment (i = 1 to 5), R_j is the random effect of run (j = 1 to 3; $R_j \sim N(0, \sigma^2_r)$), and ε_{ijk} is the random residual error. The six bottles incubated per treatment within each run (k = 1 to 6) were true experimental replicates rather than pseudoreplicates; although they shared the same rumen fluid inoculum, they were prepared, incubated, and sampled independently, and bottle-to-bottle variation within each treatment × run combination was captured in ε_{ijk}, the residual term of the model, so treatment comparisons appropriately reflect uncertainty at the level of the individual incubation bottle nested within run. Treatment means were compared using Tukey's honestly significant difference (HSD) test. Statistical significance was declared at $P < 0.05$, and tendencies were noted at $0.05 \leq P < 0.10$. Run (i.e., inoculum source) was fitted as a random blocking effect, since all five treatments were evaluated within each of the three independent runs to account for between-animal variability in rumen fluid composition. Data normality was confirmed with the Shapiro-Wilk test prior to parametric analysis. All percentage data were arc-sine transformed before analysis; back-transformed means are presented in all tables for ease of interpretation. Coefficient of variation was calculated for each response variable as an indicator of data consistency across incubation runs.

3. Results

3.1. Gas production kinetics and true dry matter digestibility

The effects of dietary treatment on gas production kinetics and TDMD are presented in Table 2. The asymptotic gas production potential (*b*) was significantly affected by treatment (P = 0.016). The control and AHP+ diets recorded equivalent and highest asymptotic gas production potential (*b*) values (149.3 and 149.2 mL/g DM, respectively), which did not differ statistically from AHP– (144.5 mL/g DM). AHT– (140.4 mL/g DM) and AHT+ (141.1 mL/g DM) recorded the lowest *b* values, significantly below those of the control and AHP+ (P < 0.05). Yeast supplementation improved *b* by 3.3% in AHP diets and by 0.5% in AHT diets, neither difference reaching statistical significance within hull origins. The fractional fermentation rate (*c*) was not significantly affected by treatment (P = 0.590), ranging from 0.087/h in the control to 0.097/h in AHP–.

The discrete lag time (Lag) was significantly influenced by treatment (P = 0.003). The control exhibited the longest Lag (2.27 h), whereas AHT– recorded the shortest (1.89 h), which was 16.7% lower than the control. Gas production at 8 h (G8), 12 h (G12), and 24 h (G24) were significantly affected by treatment (P = 0.026, 0.011, and 0.003, respectively). AHP+ produced the highest G8 (77.2 mL/g DM), G12 (99.2 mL/g DM), and G24 (132.3 mL/g DM), while AHT– and AHT+ were consistently lowest. Gas production at 4 h (G4) showed a trend (P=0.063), with AHP+ highest (45.6 mL/g DM). TDMD followed the same pattern (P=0.003): AHP+ was highest (814 g/kg DM), significantly exceeding AHT– (779 g/kg DM) and AHT+ (786 g/kg DM), representing a 4.5% improvement. The control (807 g/kg DM) and AHP– (795 g/kg DM) were intermediate.

Table 2. Effect of *Pichia manshurica* yeast supplementation on in vitro rumen gas production kinetics and true dry matter digestibility of diets containing almond hulls from Palestine or Tunisia.

Treatment	*b* (mL/g DM)	*c* (/h)	Lag (h)	G4	G8	G12	G24	TDMD (g/kg DM)
Control	149.3^{a}	0.087	2.27^{a}	43.8	74.7^{ab}	96.4^{ab}	130.5^{ab}	807^{ab}
AHP(–)	144.5^{ab}	0.097	2.13^{ab}	43.8	74.2^{ab}	95.4^{abc}	127.6^{bc}	795^{bc}
AHP(+)	149.2^{a}	0.092	2.11^{ab}	45.6	77.2^{a}	99.2^{a}	132.3^{a}	814^{a}
AHT(–)	140.4^{b}	0.088	1.89^{c}	41.8	71.1^{b}	91.7^{c}	125.4^{c}	779^{c}
AHT(+)	141.1^{b}	0.093	2.02^{bc}	43.5	73.5^{b}	94.2^{bc}	123.5^{c}	786^{c}
SEM	2.26	0.0027	0.064	0.883	1.260	1.41	1.62	6.6
P-value	0.016	0.590	0.003	0.063	0.026	0.011	0.003	0.003

AHP: almond hulls from Palestine; AHT: almond hulls from Tunisia; (–): without yeast; (+): with *Pichia manshurica* yeast (1.4×10^8 CFU/g DM per strain). *b*: asymptotic gas production potential from the insoluble fermentable fraction (mL/g DM); *c*: fractional rate constant (/h); Lag: discrete lag time (h); G4, G8, G12, G24: cumulative gas production at 4, 8, 12, 24 h (mL/g DM); TDMD: true dry matter digestibility (g/kg DM). SEM: standard error of the mean. Values in the same column with different letters differ significantly ($P < 0.05$).

3.2. Fermentation measurements

Treatment effects on ME, PF, MBP, and GY_{24} are presented in Table 3. Metabolizable energy was significantly affected by treatment ($P < 0.001$). The control diet produced the highest ME (6.7 MJ/kg DM). AHP+ was intermediate (6.3 MJ/kg DM), and AHP–, AHT–, and AHT+ were lowest (6.2, 6.1, and 6.1 MJ/kg DM, respectively). Compared to the control, ME declined by 5.4% in AHP+ and by 8.2 to 9.1% in AHT diets. The PF was significantly different among treatments ($P = 0.003$): AHT– and AHT+ recorded the highest values (6.3 mg truly degraded DM/mL gas), while the control and AHP+ shared the lowest (6.2 mg/mL). MBP was significantly affected by treatment ($P = 0.003$). AHP+ yielded the highest MBP (523.3 mg/g DM), significantly exceeding AHT– (506.9 mg/g DM) and AHT+ (510.5 mg/g DM). Compared with AHT–, AHP+ increased MBP by 3.2%. GY_{24} followed a parallel pattern ($P = 0.003$), with AHP+ (162.4 mL/g truly degraded DM) and the control (161.6 mL/g truly degraded DM) producing the most gas per unit substrate, while AHT– produced the least (158.6 mL/g truly degraded DM).

3.3. Volatile fatty acid profile

Treatment effects on VFA concentrations and molar proportions are presented in Table 4. Total VFA concentration was significantly influenced by treatment ($P = 0.007$). AHP+ produced the highest total VFA (129.6 mmol/L), representing a 37.6% increase over the control (94.2 mmol/L) and a 21.1% increase over AHP– (107.0 mmol/L). AHT+ yielded 114.8 mmol/L, a 21.9% increase over the control, whereas AHT– recorded the lowest total VFA (89.9 mmol/L, 4.6% below the control). Acetate proportion was significantly affected by treatment ($P = 0.008$). The control and AHP– maintained the highest acetate values (59.6 and 57.3%), while AHP+ had the lowest (46.1%), representing a 22.6% reduction relative to the control. Propionate proportion was significantly different ($P = 0.001$). AHP+ achieved the highest propionate (35.4%), exceeding the control by 67.8% and AHP– by 40.5%. AHT diets were intermediate (AHT–: 28.4%; AHT+: 30.2%). Butyrate was not significantly different ($P = 0.204$;

range: 14.7–18.6%). Valeric acid was significantly affected (P = 0.025), with AHP– (0.55%) and AHP+ (0.50%) higher than the control (0.15%) and AHT diets (0.11–0.15%). Isobutyric acid differed significantly (P = 0.007), with AHT+ recording a markedly lower proportion (0.02%) than all other treatments.

Table 3. Effects of *Pichia manshurica* yeast supplementation on metabolizable energy, partitioning factor, microbial biomass production, and gas yield at 24 h in diets containing almond hulls from Palestine or Tunisia.

Treatment	ME (MJ/kg DM)	PF (mg/mL)	MBP (mg/g DM)	GY24 (mL/g truly degraded DM)
Control	6.67^{a}	6.18^{b}	519.9^{ab}	161.6^{a}
AHP(–)	6.18^{c}	6.24^{ab}	514.6^{bc}	160.4^{ab}
AHP(+)	6.31^{b}	6.16^{b}	523.3^{a}	162.4^{a}
AHT(–)	6.06^{c}	6.31^{a}	506.9^{c}	158.6^{b}
AHT(+)	6.12^{c}	6.27^{a}	510.5^{c}	159.4^{b}
SEM	0.044	0.027	3.03	0.70
P-value	<0.001	0.003	0.003	0.003

AHP: almond hulls from Palestine; AHT: almond hulls from Tunisia; (–): without yeast; (+): with *Pichia manshurica* yeast (1.4×10^8 CFU/g DM per strain); ME: metabolizable energy (MJ/kg DM); PF: partitioning factor (mg truly degraded DM/mL gas); MBP: microbial biomass production (mg/g DM); GY_{24}: gas yield at 24 h (mL/g truly degraded DM). SEM: standard error of the mean. Values in the same column with different letters differ significantly (P < 0.05).

Table 4. Effects of *Pichia manshurica* yeast supplementation on volatile fatty acid profiles during in vitro rumen incubation of diets containing almond hulls from Palestine or Tunisia.

Treatment	Total VFA (mmol/L)	Acetic (A; %)	Propionic (P; %)	A:P ratio	Butyric (%)	Valeric (%)	Isovaleric (%)	Isobutyric (%)
Control	94.2^{c}	59.6^{a}	21.1^{c}	2.82	18.6	0.15^{b}	0.07	0.51^{a}
AHP(–)	107.0^{bc}	57.3^{a}	25.2^{bc}	2.27	16.2	0.55^{a}	0.33	0.45^{a}
AHP(+)	129.6^{a}	46.1^{b}	35.4^{a}	1.30	17.6	0.50^{a}	0.12	0.35^{a}
AHT(–)	89.9^{c}	53.7^{a}	28.4^{b}	1.89	17.3	0.11^{b}	0.08	0.44^{a}
AHT(+)	114.8^{ab}	54.6^{a}	30.2^{b}	1.81	14.7	0.15^{b}	0.37	0.02^{b}
SEM	6.05	2.02	1.611	0.083	1.117	0.107	0.102	0.08
P-value	0.007	0.008	0.001	0.003	0.204	0.025	0.150	0.007

AHP: almond hulls from Palestine; AHT: almond hulls from Tunisia; (–): without yeast; (+): with *Pichia manshurica* yeast (1.4×10^8 CFU/g DM per strain). Total VFA: total volatile fatty acids (mmol/L); acetic, propionic, butyric, valeric, isovaleric, and isobutyric acids expressed as molar proportions (% of total VFA). SEM: standard error of the mean. Values in the same column with different letters differ significantly (P < 0.05).

3.4. Ruminal pH, methane production, nutrient digestibility, and protozoal counts

Ruminal pH was not significantly different among treatments (P = 0.482), although AHT– had the numerically lowest pH (4.9) compared to the control (5.7) and other treatments (5.5–5.6). Methane production per gram of DM differed significantly among treatments (P = 0.039) (Table 5). The control

produced the least CH_4 (21.9 mL/g DM), while AHP+ produced the most (32.6 mL/g DM), a 48.9% increase. AHT– and AHT+ produced 30.5 and 30.8 mL/g DM, both significantly higher than the control. CH_4/TDOM was not significantly different (P = 0.059; range: 27.9–40.8 mL/g TDOM). CH_4/TNDF was significantly affected (P = 0.004): The control was lowest (40.7 mL/g TNDF) and all AH diets were markedly higher (72.4–77.5 mL/g TNDF).

Table 5. Effects of *Pichia manshurica* yeast supplementation on ruminal pH, methane production, organic matter and NDF digestibility, and protozoal counts during in vitro incubation of diets containing almond hulls from Palestine or Tunisia.

Item	Control	AHP(–)	AHP(+)	AHT(–)	AHT(+)	SEM	P-value
pH	5.7	5.6	5.5	4.9	5.6	0.34	0.482
CH_4 (mL/g DM)	21.9^{b}	25.6^{ab}	32.6^{a}	30.5^{a}	30.8^{a}	2.47	0.039
TDOM (g/kg DM)	786.4^{c}	777.2^{c}	799.5^{b}	812.9^{ab}	813.8^{a}	4.95	0.001
CH_4/TDOM (mL/g)	27.9	32.9	40.8	37.5	37.9	3.11	0.059
TNDF (g/kg NDF)	539.1^{a}	357.4^{c}	421.5^{b}	411.1^{b}	416.0^{b}	14.49	<0.001
CH_4/TNDF (mL/g)	40.7^{b}	72.4^{a}	77.5^{a}	74.4^{a}	74.5^{a}	6.99	0.004
Protozoa ($\times 10^5$/mL)	10.1	17.9	13.2	10.5	10.95	2.34	0.155

AHP: almond hulls from Palestine; AHT: almond hulls from Tunisia; (–): without yeast; (+): with *Pichia manshurica* yeast (1.4×10^8 CFU/g DM per strain). CH_4: methane production (mL/g DM); TDOM: true digestible organic matter (g/kg DM); CH_4/TDOM: methane per unit of truly digested organic matter (mL/g TDOM); TNDF: true NDF degradability (g/kg NDF); CH_4/TNDF: methane per unit of truly digested NDF (mL/g TNDF); Protozoa: protozoal count ($\times 10^5$/mL). SEM: standard error of the mean. Values in the same row with different letters differ significantly (P < 0.05).

True digestible organic matter (TDOM) was highly significantly affected by treatment (P = 0.001). AHT+ and AHT– achieved the highest TDOM (813.8 and 812.9 g/kg DM, respectively), both significantly exceeding the control (786.4 g/kg DM), and AHP– (777.2 g/kg DM), with AHT+ also exceeding AHP+ (799.5 g/kg DM). Compared to the control, AHT+ increased TDOM by 3.5%. TNDF was also highly significantly affected (P < 0.001). The control recorded the highest TNDF (539.1 g/kg NDF), while all AH diets were substantially lower (357.4–421.5 g/kg NDF). Within AHP diets, yeast supplementation improved TNDF from 357.4 g/kg DM (AHP–) to 421.5 g/kg NDF (AHP+), a 17.9% increase. Protozoal counts were not significantly different among treatments (P = 0.155), although AHP– recorded the numerically highest count (17.9×10^5/mL) compared to the control (10.1×10^5/mL). Yeast supplementation numerically reduced protozoal counts in AHP+ (13.2×10^5/mL) relative to AHP–.

4. Discussion

4.1. Gas production kinetics and true dry matter digestibility

The significantly lower asymptotic gas production potential (*b*) in AHT diets relative to the control and AHP+ is consistent with the chemical composition differences between the two hull origins. Palestinian almond hulls had higher NDF (31.23 vs. 28.79% for AHT), higher ADL (3.32 vs. 2.38%), and higher hemicellulose content (20.18 vs. 17.89%), providing a larger pool of structurally complex but fermentable carbohydrates. Zoabi et al. [1], who compared almond hulls from these two origins

using *in vitro* fermentation, similarly reported that Tunisian hulls had higher fiber concentrations and produced different fermentation profiles. The present numerical comparisons among treatments are consistent with these origin-related differences: AHP+ maintained *b* equivalent to the berseem control, while AHT+ showed only a marginal, non-significant improvement.

The apparent difference in yeast responsiveness between hull origins, based on the numerical comparisons described above rather than a formally tested interaction term, aligns with the proposed mechanism by which yeast stimulates fermentation. *P. manshurica*, like other yeasts with oxygen-scavenging capacity, creates a more favorable microenvironment for strict anaerobic cellulolytic bacteria such as *Ruminococcus champanellensis* and *Fibrobacter succinogenes* [9]. These organisms preferentially colonize complex, insoluble cell wall structures, making their stimulation more impactful when the substrate contains abundant fermentable fiber. The higher NDF and hemicellulose content of AHP thus provides greater opportunity for yeast-mediated cellulolytic enhancement than the lower-fiber AHT matrix. Soltan et al. [13] demonstrated that the same *P. manshurica* strains used here stimulate fiber degradation in mixed rumen cultures by supporting cellulolytic bacterial populations, providing direct mechanistic support for the greater yeast response observed in AHP+ compared to AHT+. Furthermore, the lower ADL of AHT hulls (2.38%) versus AHP (3.32%) and berseem (3.82%) reduces the indigestible cell wall fraction, which would normally facilitate digestibility; the paradoxically lower *b* in AHT diets therefore likely reflects a substrate quantity effect rather than an inhibitory one.

The absence of a significant yeast effect on the fractional fermentation rate (*c*) is consistent with prior in vitro evidence. Phesatcha et al. [26] reported that *Saccharomyces cerevisiae* supplementation at a 60:40 roughage-to-concentrate ratio, identical to that used in this study, improves fermentation extent without altering the fractional rate. Wang et al. [7] similarly found that yeast culture increased total VFA and improved substrate degradation magnitude in sheep diets without accelerating the fractional rate constant. These consistent findings across yeast species and substrate types suggest that yeast primarily expands the fermentable substrate pool accessible to the existing microbial community rather than increasing the intrinsic enzymatic activity rate [7,26]. The TDMD findings corroborate the gas production data, with AHP+ achieving 81.4%, equivalent to the control (80.7%) and 4.5% above AHT– (77.9%), confirming a meaningful nutritional advantage for Palestinian hull diets supplemented with *P. manshurica*.

The significantly shorter Lag in AHT– (1.89 h) compared with the control (2.27 h) merits interpretation. A shorter lag phase implies that microbial colonization of the substrate surface and initiation of fermentation activity occur more rapidly in AHT– than in other treatments. This may reflect the lower ADL content of Tunisian hulls (2.38%), which reduces the physical barrier to microbial enzyme access, enabling rapid fermentation of surface-exposed non-structural carbohydrates before the more recalcitrant fiber fractions are engaged [27]. However, the shorter lag did not translate into greater total fermentation, as AHT– produced the lowest *b* value (140.4 mL/g DM), confirming that rapid initial fermentation onset does not predict total substrate fermentability. This divergence between Lag and *b* is well recognized in the in vitro gas production literature and emphasizes the importance of reporting the full kinetic parameter set rather than relying on single endpoints. Yeast supplementation had no significant effect on Lag in either AHP or AHT diets, suggesting that the initial microbial colonization dynamics are governed by substrate surface chemistry and particle size rather than by yeast-mediated metabolic facilitation.

4.2. Fermentation measurements

The reduction in ME across all almond hull diets relative to the berseem control reflects the combined effects of lower crude protein (approximately 9.0 vs. 16.24%) and lower 24-h gas production (G24) in AH-based diets. The Menke et al. [21] equation integrates both parameters, making the ME differential partly a consequence of the lower crude protein content of almond hulls independent of fermentability. AHP+ recovered the most ME among AH treatments (6.3 MJ/kg DM) by virtue of its superior G24 (132.3 mL/g DM). The 5.4% ME deficit of AHP+ versus the control may be partially offset in practice by the substantially greater total VFA output (37.6% increase), which represents the primary energy currency absorbed from the rumen. Liu et al. [28], evaluating active dry yeasts in lambs, similarly observed ME recovery with Vistacell yeast supplementation that improved NDF digestibility and propionate production, mitigating ME losses otherwise observed in by-product-supplemented diets.

Microbial biomass production (MBP) is a key determinant of metabolizable protein supply in ruminants, since the bulk of absorbable amino acids reaching the small intestine of forage-fed animals originates from microbial protein rather than from dietary protein escaping ruminal degradation [29]. AHP+ achieved the highest MBP (523.3 mg/g DM), significantly exceeding all AHT treatments (506.9–510.5 mg/g DM). The comparatively lower MBP in AHT diets, despite their higher TDOM, parallels their lower total VFA output and propionate proportion and reflects the weaker yeast-substrate synergy noted for Tunisian hulls; because AHT hulls contain less structurally complex fiber for cellulolytic bacteria to colonize, the fermentable substrate pool available for yeast-stimulated microbial growth is comparatively smaller, limiting the scope for yeast-driven MBP enhancement even though overall TDOM is higher. This finding is nutritionally important because replacing the protein-rich berseem hay forage component (crude protein: 16.24% of the mixed diet DM) with almond hulls (crude protein: approximately 9.0% of the mixed diet DM) inherently reduces dietary nitrogen supply of the mixed ration; a concurrent yeast-mediated improvement in MBP would partially compensate for this reduction by increasing the efficiency with which the available nitrogen pool is captured into microbial cells rather than lost as ammonia. Ammonia-N concentration, a direct indicator of ruminal nitrogen capture efficiency, was not measured in this in vitro study; the higher MBP in AHP+ is therefore consistent with, but does not by itself confirm, more efficient microbial nitrogen capture, and direct ammonia-N determination is recommended in future work to verify this mechanism. The relationship between propionate production and MBP in AHP+ is plausibly, though not directly, linked: The shift from acetate-dominant to propionate-dominant fermentation in AHP+ would be consistent with a more efficient carbohydrate utilization pathway, in which less metabolic hydrogen is hypothesized to be dissipated as CH_4 and more carbon and energy are channeled into microbial growth rather than into gaseous end products, enabling greater proportional retention as microbial biomass. This interpretation is consistent with the partitioning-factor framework, in which a higher ratio of truly degraded substrate to gas volume reflects more efficient microbial growth, established by Blümmel et al. [22], who demonstrated that the partitioning of fermented substrate into microbial biomass versus gaseous end-products is a key determinant of the nutritional value of fermented feeds. By this metric, the numerically higher PF in AHT diets (6.3 mg/mL) relative to the control and AHP+ (6.2 mg/mL) would formally indicate that a slightly greater proportion of truly degraded substrate was retained as biomass per unit of gas produced in AHT diets; however, this per-gas ratio must be interpreted alongside the absolute MBP values, which were lower in AHT diets than in AHP+. This apparent discrepancy likely reflects the smaller total amount of substrate fermented in AHT diets (lower *b* and G24),

so that even a marginally favorable partitioning ratio translates into less absolute microbial biomass than the more extensively fermented AHP+ diet; the PF differences among treatments, though statistically significant, were numerically small (6.2 vs. 6.3 mg/mL) and should therefore be interpreted with caution.

The MBP values obtained in this study are within the range reported for similar in vitro systems. Nueraihemaiti et al. [30], who evaluated two self-developed yeast cultures versus a commercial product in Hu sheep diets in vitro, reported MBP improvements of similar relative magnitude with the more effective yeast treatment (comparable in magnitude to our AHP+ treatment), confirming that MBP responsiveness to yeast is a consistent phenomenon across yeast species and substrate types. The concurrent improvement in GY_{24} in AHP+ (162.4 mL/g truly degraded DM) relative to AHT– (158.6 mL/g truly degraded DM) reflects the greater total fermentation efficiency of the yeast-supplemented Palestinian hull diet, providing further evidence that the combination of AHP and *P. manshurica* is associated with a combined improvement in rumen microbial productivity beyond that of the control diet, although this study was not designed to formally test yeast × hull-origin interaction effects. From a feed formulation standpoint, the GY24 and MBP data collectively suggest that replacing berseem with AHP at 1.4×10^8 CFU/g DM per *P. manshurica* strain preserves the fermentative nutritive value of the diet for ruminants, whereas AHT diets, even with yeast supplementation, represent a nutritional step-down relative to the berseem baseline.

4.3. Volatile fatty acid profile

The 37.6% increase in total VFA in AHP+ relative to the control (129.6 vs. 94.2 mmol/L) represents one of the most notable in vitro findings of this study, though its practical significance in vivo remains to be confirmed. Total VFA production is the primary determinant of energy availability from rumen fermentation, and such a magnitude of increase indicates a major improvement in substrate utilization efficiency when *P. manshurica* yeast is combined with Palestinian almond hulls. This finding likely reflects the combined oxygen-scavenging action of the yeast, which creates conditions favorable to strictly anaerobic cellulolytic bacteria, together with the provision of B vitamins, nucleotides, and metabolic cofactors that stimulate microbial activity. Wang et al. [7] reported comparable increases in total VFA with yeast culture supplementation in high-concentrate sheep diets, attributing the effect to shifts in bacterial community composition toward *Prevotella*-dominated configurations associated with propionate synthesis. The 21.9% increase in total VFA in AHT+ compared to the control further confirms that *P. manshurica* confers a meaningful fermentation benefit even in the Tunisian hull context, despite the weaker substrate-yeast compatibility compared to AHP diets.

The pronounced propionate shift in AHP+, from 21.1% in the control to 35.4% (a 67.8% increase), is nutritionally and metabolically important. Propionate is the principal gluconeogenic substrate in ruminants, supplying up to 50% to 60% of the glucose used for hepatic gluconeogenesis in high-producing animals [31]. The A:P ratio reduction from 2.82 in the control to approximately 1.30 in AHP+ indicates a fundamentally different fermentation pattern that would be expected to improve energy efficiency in vivo. Liu et al. [28] confirmed that Vistacell active dry yeast increased propionate proportion and reduced n-butyrate in Hu lambs in vitro and in vivo, suggesting that the propionogenic shift observed here in vitro may have analogous effects in vivo, although this would need to be confirmed directly in this dietary context. The mechanism involves competitive hydrogen utilization

via the succinate and acrylate pathways of propionate synthesis, which compete with methanogenesis for reducing equivalents, a dynamic consistent with the concurrent CH_4 findings discussed below. Soltan et al. [13] also reported increased propionate with the same *P. manshurica* strains, confirming the propionogenic tendency of these yeasts across substrate types.

The significant reduction in acetate proportion in AHP+ (46.1% vs. 59.6% in control) without a significant change in butyrate indicates a selective re-routing of fermentation products toward propionate rather than a generalized disruption of the fermentative microbiome. Butyrate proportions were maintained across treatments (14.7–18.6%), which is practically favorable because butyrate supports rumen epithelial development and proliferation of ruminal papillae. The elevation of valeric acid in AHP– and AHP+ relative to other treatments may reflect enhanced proteolytic or amino acid-fermentative activity associated with the Palestinian hull diet matrix. This interpretation is plausible but not directly confirmed in this study, as ammonia-N and proteolytic enzyme activity were not measured; it should therefore be regarded as a hypothesis warranting direct testing rather than an established mechanism. The near-complete suppression of isobutyric acid in AHT+ (0.02% vs. 0.35–0.51% in other treatments) is a singular observation that may indicate a treatment-specific community shift; however, given the absence of microbiome characterization data, its interpretation remains preliminary.

Taken holistically, the VFA data strongly support the nutritional superiority of AHP+ among the almond hull-based treatments. These combined increases in total VFA and propionate proportion indicate that *P. manshurica* yeast combined with Palestinian almond hull fiber is associated with markedly improved fermentation outcomes relative to either component's expected individual contribution, although a formal factorial design would be needed to confirm true synergism. The practical implication is that ruminant producers in regions where Palestinian almond hulls are abundant can substitute these hulls for berseem clover at a 60:40 forage-to-concentrate ratio with limited apparent loss of fermentable energy provision under the in vitro conditions tested here, pending confirmation in vivo, provided yeast is co-administered at the tested dose of 1.4×10^8 CFU/g DM per strain. For Tunisian almond hull diets, yeast supplementation confers meaningful VFA improvements (21.9% above control in AHT+), though the overall fermentation profile remains inferior to AHP+ in terms of total VFA and propionate output. These differences in yeast responsiveness between hull origins have direct implications for the design of precision feeding strategies in Mediterranean ruminant production systems.

4.4. Methane production and fiber digestibility

The significantly higher CH_4 per gram of DM in AHP+, AHT–, and AHT+ relative to the control is plausibly related to the substantially increased total fermentation activity, particularly the 37.6% increase in total VFA in AHP+, rather than to a specific stimulation of methanogenic archaea. Although propionate synthesis competes with methanogenesis for metabolic hydrogen, in AHP+, the large absolute increase in total fermentable substrate degraded appears to outweigh this competitive effect, so that even with the highest propionate proportion among treatments, AHP+ still generates more total hydrogen, and therefore more absolute CH_4 than the less extensively fermented control diet. Because CH_4 arises as a stoichiometric byproduct of microbial hydrogen production during fermentation, greater total substrate fermentation inevitably generates more absolute CH_4. This interpretation is consistent with the non-significant treatment effect on CH_4/TDOM, rather than

reflecting a specific effect on methanogenesis. Because this comparison did not reach the threshold for statistical significance, it should be regarded as not providing clear evidence of a treatment difference once CH_4 is standardized to the amount of substrate fermented, rather than as demonstrating statistical equivalence among treatments. These results contrast with Soltan et al. [13], who reported a 23.5% CH_4 reduction with the same *P. manshurica* Mix in aflatoxin-contaminated diets; the discrepancy likely reflects substrate differences, as the fibrous low-starch almond hull matrix generates more hydrogen from cellulose fermentation, potentially overwhelming any methanogen-suppressing effect of the yeast. Malyugina et al. [32] emphasized that yeast effects on CH_4 are highly context-dependent (i.e., varying with diet composition, yeast dose, and animal factors) and substrate fermentability is the dominant modulating factor, which is fully consistent with our outcomes.

The markedly elevated CH_4/TNDF in all AH diets relative to the control (72.4–77.5 vs. 40.7 mL/g TNDF) does not indicate inherently greater methanogenic efficiency of almond hull diets, but rather reflects the substantially lower TNDF of AH-based diets compared to the berseem control. The control's high TNDF (539.1 g/kg NDF) means that the same absolute CH_4 output is expressed against a much larger digestibility denominator, yielding a lower ratio. The berseem hay, with its high NDF content (54.2%) and legume-adapted microbial community, naturally supports efficient fiber fermentation; this advantage is not replicated by almond hulls regardless of yeast supplementation. Zoabi et al. [1], studying almond hulls from the same two origins with chemical treatments, observed similar differences in fiber digestibility between Palestinian and Tunisian hulls, confirming that origin-specific hull chemistry fundamentally constrains the fermentative potential achievable in vitro.

The 17.9% improvement in TNDF achieved by yeast supplementation in AHP diets (357.4 to 421.5 g/kg NDF) is a practically significant finding, demonstrating that *P. manshurica* yeast can partially overcome the fiber fermentability constraints of Palestinian almond hull diets by supporting cellulolytic microbial activity. This effect was less pronounced in AHT diets (411.1 to 416.0 g/kg NDF), consistent with the interpretation that the Tunisian hull matrix provides less substrate for yeast-facilitated cellulolytic enhancement. Zoabi et al. [1], who evaluated almond hulls from the same two origins (Palestine or Tunisia), reported that treating the hulls with urea increased NDF and acid detergent fiber degradability by disrupting cell wall cross-links, suggesting that chemical pre-treatment may synergize with yeast supplementation to further enhance fiber digestibility in AHT diets, a combined strategy that warrants direct experimental evaluation. It is also plausible that increasing the *P. manshurica* dose beyond the 1.4×10^8 CFU/g DM per strain used here could further improve fermentation of the less fiber-complex Tunisian hull matrix; however, this would need to be verified experimentally through a dose-response trial, since higher yeast doses do not always translate into proportionally greater fermentation responses and may be constrained by substrate availability rather than yeast concentration. Thus, future in vivo studies should quantify total daily enteric CH_4 output per animal to determine whether the enhancement of fermentation efficiency and MBP in AHP+ diets translates to an acceptable CH_4 efficiency tradeoff under practical production conditions. The environmental impact of adopting almond hull-based diets with yeast supplementation should be assessed on an intensity basis (CH_4 per unit of animal product) rather than per unit of DM, as the substantial gains in fermentation productivity in AHP+ may reduce the CH_4 intensity of production despite the elevated absolute CH_4 per gram of DM.

4.5. Ruminal pH, organic matter digestibility, and protozoal population

The non-significant treatment effect on ruminal pH is attributable to the buffered in vitro system, which maintains pH within a physiologically plausible range. Because the Menke and Steingass buffer provides substantial exogenous bicarbonate buffering capacity independent of yeast presence, it also stabilizes pH in the non-yeast-supplemented treatments (AHP– and AHT–), which is why no significant pH difference emerged between yeast and non-yeast diets in this in vitro system; under in vivo conditions, where buffering relies primarily on saliva rather than an exogenous buffer, the non-yeast-supplemented treatments would be expected to show a more pronounced pH decline than observed here. Nevertheless, the numerically lower pH in AHT– (4.9) compared to all other treatments (5.5–5.7) is noteworthy. A ruminal pH of 4.9 would be indicative of acidotic conditions if it occurred in vivo [11,12] and could adversely affect cellulolytic bacteria whose optimal pH range is 6.0–6.8; because this pH difference did not reach statistical significance in the buffered system, it should not be interpreted as demonstrating in vivo acidotic risk. Numerically, pH was higher in AHT+ (5.6) than in AHT– (4.9); although this single buffered in vitro comparison does not by itself establish yeast-mediated pH stabilization, oxygen-scavenging and lactate-consuming mechanisms of this general kind have been proposed for *S. cerevisiae* [33] and now extended to *P. manshurica*, although this mechanism was not directly tested for *P. manshurica* in our in vitro almond hull system. Baker et al. [8] similarly concluded that yeast supplementation contributes to rumen pH stabilization through its support of lactate-utilizing bacteria. Given the non-significant treatment effect on pH in the present buffered in vitro system, the numerical pattern observed here is only broadly consistent with, and does not independently confirm, this proposed mechanism.

The pH finding in AHT– has a direct fermentation consequence: The numerically suppressed pH of 4.9 in that treatment is consistent with the lowest total VFA concentration (89.9 mmol/L), lowest *b* value (140.4 mL/g DM), and lowest TDMD (77.9%) recorded in the study. Although the pH effect did not reach statistical significance, likely because the bicarbonate buffer partially neutralized acid accumulation, the convergence of these unfavorable fermentation outcomes in AHT– suggests that uncontrolled fermentation of Tunisian almond hull carbohydrates without yeast may predispose the rumen environment to acidogenic conditions. Under in vivo conditions, where buffering capacity is provided by saliva rather than an exogenous buffer, this acidogenic potential could have more pronounced effects on fiber-degrading populations and overall feed efficiency. Because this pH difference was not statistically significant in our buffered in vitro system, this numerical pattern should be regarded as preliminary and would need to be confirmed under in vivo conditions before practical significance can be inferred.

The highest TDOM values in AHT– and AHT+ (812.9 and 813.8 g/kg DM) versus the control (786.4 g/kg DM), a 3.5% difference, reflect the lower ADL content of Tunisian hulls (2.38% vs. 3.82% in the control diet), which reduces the indigestible cell wall fraction and frees a greater proportion of organic matter for microbial attack. Rad et al. [2] similarly reported that the replacement of alfalfa with almond hulls in fattening lamb diets did not compromise in vivo OM digestibility, attributing this to the comparatively low lignification of almond hull material. Zoabi et al. [1] also reported that NaOH and urea treatments of Tunisian almond hulls further increased TDOM by disrupting cell wall linkages (although those hulls were evaluated as chemically treated hulls without live-yeast supplementation, unlike the untreated, yeast-supplemented hulls used in this study), confirming that the inherently low lignin content of AHT already confers high organic matter accessibility. It is important to note that the

high TDOM of AHT diets coexists with their lower total VFA output, MBP, and gas production relative to AHP+ diets, indicating that organic matter digestibility alone does not fully capture the nutritional superiority of a dietary treatment.

Protozoal counts were not significantly affected by treatment. The numerically elevated count in AHP– (17.9×10^5/mL) compared to the control (10.1×10^5/mL) and the partial reduction in AHP+ (13.2×10^5/mL) suggest that yeast supplementation may exert a modest anti-protozoal effect. Protozoa are predators of ruminal bacteria and can reduce net microbial protein flow to the duodenum [34]; their numerically lower count in AHP+ versus AHP– is therefore consistent with the higher MBP observed in AHP+. Although the higher-quality berseem control forage would intuitively be expected to yield the highest microbial biomass, AHP+ instead achieved the numerically highest MBP overall (523.3 vs. 519.9 mg/g DM in the control); this is attributable to the markedly more propionate-dominant fermentation pattern in AHP+, which channels a greater share of fermented carbon and metabolic hydrogen into microbial growth rather than into gaseous end products. Majewska et al. [35] reported reduced *Diplodinium* spp. abundance following *S. cerevisiae* supplementation in sheep, and Lila et al. [36] observed non-significant protozoal changes across substrates with twin-strain yeast supplementation, a finding consistent with the result of this experiment.

The elevated protozoal count in AHP may be related to the characteristics of the almond hull substrate. Palestinian almond hull material, with its higher hemicellulose content (20.18% vs. 17.89% for AHT), may provide particularly fermentable non-structural carbohydrate fractions that support rapid protozoal proliferation in the absence of yeast. It should be noted that total VFA concentration in AHP– (107.0 mmol/L) was not the highest among all treatments, so this hypothesis rests specifically on the composition of readily fermentable carbohydrate (hemicellulose-derived sugars) rather than on overall fermentation extent and remains to be confirmed by direct measurement of protozoal substrate preference. Protozoa are known to preferentially sequester and digest starch and soluble sugars, a generally established feature of rumen protozoal metabolism [34], and hemicellulosic sugars released during fermentation may partially fulfill this role. However, protozoal counts did not differ significantly among treatments, and these numerical patterns should therefore be regarded as preliminary. In vivo studies with extended supplementation periods, coupled with protozoal species-level identification, are required to clarify whether the numerically observed anti-protozoal tendency in AHP+ translates to a statistically and biologically meaningful effect in live animals.

5. Conclusions

Supplementation of almond hull-based diets with rumen-native *P. manshurica* strains FFNLYFC1 and FFNLYFC2 (1.4×10^8 CFU/g DM per strain) selectively improved specific in vitro rumen fermentation variables, namely total VFA concentration and propionate proportion in both hull origins, and, in Palestinian hull diets specifically, gas production potential, true dry matter digestibility, true NDF degradability, and microbial biomass production; these improvements were not uniform across all measured variables or both hull origins, and were accompanied by higher absolute methane production per gram of DM in the yeast-supplemented diets. In Palestinian almond hull diets, yeast supplementation maintained gas production potential and TDMD comparable to the berseem clover control while markedly enhancing fermentation characteristics, including total VFA concentration, propionate proportion, TNDF, and microbial biomass production. These results indicate that the combination of Palestinian almond hulls with *P. manshurica* can effectively preserve or exceed several

in vitro fermentation characteristics of a conventional berseem clover-based ration; because the almond hull diets had lower crude protein and estimated ME than the control, this in vitro fermentation advantage should not be extrapolated to overall dietary nutritional adequacy without in vivo confirmation. Tunisian almond hull diets exhibited greater TDOM values, reflecting lower lignification, but produced lower VFA concentrations and fiber degradation than Palestinian almond hull diets. Nevertheless, yeast supplementation remained beneficial, increasing total VFA production relative to the unsupplemented control. Collectively, these findings support the use of Palestinian almond hulls supplemented with *P. manshurica* as a promising partial forage replacement for ruminants in 60:40 forage-to-concentrate diets under the in vitro conditions tested, warranting in vivo evaluation of overall nutritional adequacy before practical recommendations can be made while promoting the valorization of locally available agro-industrial by-products. In future studies, researchers should validate these findings under in vivo conditions, assess impacts on animal productivity and feed efficiency, characterize rumen microbial responses using metagenomic approaches, evaluate dose-response relationships in Tunisian almond hull diets, and quantify enteric methane emissions. Our results further highlight the importance of explicitly considering and reporting almond hull origin in nutritional studies, as origin-specific chemical composition substantially influences the ruminal fermentation response to yeast supplementation.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

HZ: Conceptualization, Data collection, Methodology, Investigation, Data curation; YAS: Conceptualization, Data collection, Methodology, Investigation, Data curation; ASM: Conceptualization, Data collection, Methodology, Investigation,, Data curation; HME: Conceptualization, Data collection, Methodology, Investigation, Data curation; AEK: Conceptualization, Methodology, Data curation, Original Draft Preparation, Writing – Reviewing and Editing, Writing – Final Draft Preparation, Formal Analysis; RBR: Investigation, Data curation, Writing – Reviewing and Editing; SG: Writing – Original Draft Preparation, Writing – Reviewing and Editing, Writing – Final Draft Preparation, SH: Conceptualization, Data curation, Writing – Final Draft Preparation; BAA: Data collection, Original Draft Preparation, Writing – Final Draft Preparation; JJ: Original Draft Preparation, Data curation, Writing – Final Draft Preparation. HA: Conceptualization, Methodology, Investigation, Data curation, Writing – Original Draft Preparation, Writing – Final Draft Preparation, Supervision.

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