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Biotechnological Valorization of Almond Hulls via Solid-State Fermentation with *Saccharomyces cerevisiae* and Fibrolytic Enzyme Supplementation: Enhancing Ruminal Fermentation and Reducing Greenhouse Gas Emissions

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Abstract

Valorization of agricultural by-products is a key component of circular strategies aimed at enhancing the sustainability of livestock systems. Almond hulls (AHs), a major residue of the almond-processing industry, are characterized by their high non-fiber carbohydrate (NFC) content, but low crude protein (CP) content and ruminal fermentation. This study evaluated the effects of treating AHs with exogenous fibrolytic enzymes (EFEs) and *Saccharomyces cerevisiae* (SC) via solid-state fermentation. Treatments were applied individually or in combination (SC + EFEs). The effects on chemical composition and ruminal fermentation were assessed. EFEs reduced the fiber content and increased the NFC content. This accelerated ruminal fermentation and reduced the lag time. However, it did not change the overall fermentation extent. SC increased the CP content and ether extract but reduced the NFC content. This modification promoted the growth of ruminal bacteria. As a result, the ruminal fermentation extent, ruminal degradability and volatile fatty acid (VFA) content improved. However, methane (CH_4) and carbon dioxide (CO_2) emissions relative to the substrate, degraded substrate and total gas emission were not affected. SC + EFEs had synergistic effects. This further increased the CP content and ether extract and reduced the NFC and fiber contents. The treatment modulated ruminal microbiota by decreasing protozoa and increasing bacteria. It also reduced the fermentation lag time and enhanced the fermentation extent, degradability and VFA production favoring propionate formation. Additionally, it reduced CH_4 and CO_2 emissions per unit of degraded substrate and the total gas emission. Overall, the SC + EFEs represent an effective approach to enhance the nutritional value of AHs while partially mitigating greenhouse gas emissions relative to substrate utilization and fermentation pathways.

Keywords: almond hulls; solid-state fermentation; *Saccharomyces cerevisiae*; exogenous fibrolytic enzymes; ruminal fermentation; greenhouse gas emissions

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1. Introduction

The global almond (*Prunus dulcis*) industry has expanded rapidly over the past decade, becoming one of the most economically valuable sectors within horticulture [1]. Production is mainly concentrated in the United States and Mediterranean regions, where almond cultivation contributes significantly to rural economies and agri-food value chains [2].

During the 2023/2024 production season, global almond nut output reached approximately 1.51 million metric tons [3]. Industrial operations such as dehulling, shelling, and blanching generate residues that account for about 89% of the total fruit biomass, with almond hulls (AHs) representing the largest fraction, at approximately 63% of the fruit mass [4,5]. Globally, this equates to an estimated 3.5 million metric tons of AHs annually, much of which remains underutilized or is disposed of through landfilling or incineration, leading to considerable environmental pressures and economic inefficiencies [3,6]. Therefore, the development of sustainable valorization strategies for AHs is essential to integrate agro-industrial residues into circular bioeconomy frameworks and reduce the environmental impact [7].

AHs are characterized by high levels of soluble sugars (glucose, fructose, sucrose) and structural carbohydrates, as well as their widespread availability and low acquisition cost, making them attractive as alternative energy sources in ruminant nutrition [6,8–11]. Moreover, AHs are rich in phenolic compounds, which exhibit high antimicrobial activity against pathogens and can interact synergistically with probiotic microorganisms, thereby representing a promising natural resource for nutraceutical and pharmaceutical applications [12]. Their inclusion in ruminant diets has also been associated with the improved oxidative stability of animal-derived products, further underscoring their functional value [1,13,14] as well as reduced methane emissions [15,16]. However, their incorporation into diets has been reported to reduce the milk production and milk protein yield of cows [17] and growth performance of lambs [14] due to their low crude protein (CP) content, moderate metabolizable energy, and limited ruminal degradability, with only 50–59% of dry matter (DM) and 43–50% of neutral detergent fiber (NDF) being degraded in the rumen. These limitations constrain the inclusion of AHs in diets for high-producing ruminants, emphasizing the need for strategies to enhance their nutritive value [18–21].

To overcome the nutritional limitations of AHs, various processing strategies have been explored. Physical treatments aimed at removing foreign materials enhance fiber digestibility and energy availability [8], while chemical treatments with alkali agents promote fiber solubilization and improve ruminal fermentation efficiency [21]. However, the application of chemical treatments are often limited by environmental risks, operational hazards, and restricted scalability [22]. In this context, biotechnological approaches have gained increasing attention as environmentally sustainable and effective alternatives for the valorization of low-quality feed resources [23,24].

Among these approaches, solid-state fermentation (SSF) using probiotic microorganisms, particularly *Saccharomyces cerevisiae* (SC), has been widely established as an effective strategy to enhance the feed quality of both conventional and non-conventional feed resources. In conventional feeds such as soybean meal, SSF with SC reduces anti-nutritional factors, including phytic acid and trypsin inhibitors, while simultaneously increasing the CP content through microbial biomass synthesis and improving the profile of essential amino acids, including arginine, histidine, lysine, methionine, leucine, isoleucine, threonine, phenylalanine, and valine, and a reduced fiber content [25,26]. Similar benefits have been reported for non-conventional feed resources including complexes of corn stalk, cotton leaf, cottonseed meal, corncob, pepper meal, and molasses [27] as well as in cactus pear [28]. In vitro rumen simulation studies further demonstrate that SSF with SC significantly enhances the ruminal DM degradability (DMD) of cactus pear [28] and total volatile fatty acid (TVFA) production and nitrogen utilization of complexes of corn stalk, cotton leaf, cottonseed meal, corncob, pepper meal, and molasses [27]. In addition, in vivo studies have shown that supplementation with live SC to a high-concentrate finishing diet fed to young Charolais bulls improves feed intake, tends to reduce the number of days required to reach

optimal finishing status, prevents ruminal papillae hyperkeratinization, and enhances carcass conformation scores [29].

In parallel, exogenous fibrolytic enzymes (EFEs) have gained increasing interest as feed additives for ruminant nutrition due to the progressive reduction in their production costs, which has improved their economic feasibility [30,31] and their recognition as safe feed additives [32]. Within the rumen, EFEs accelerate the ruminal passage rate, enhance microbial attachment to plant biomass, stimulate the activity of fibrolytic microbial populations, reduce digestive fluid viscosity, and improve fiber and protein digestion. These effects collectively promote TVFA production and increase energy availability, ultimately supporting improved ruminal performance and productive responses, including increased hot carcass weight and milk production [30,33]. Recent research involving finishing steers has demonstrated that EFE supplementation enhances nitrogen metabolism by increasing the microbial utilization of ammonia for growth. This response was accompanied by shifts in ruminal fermentation patterns, characterized by increased acetate-to-propionate ratio changes in microbial community composition at the family and genus levels and increased ruminal bacterial diversity [34]. Similarly, an in vivo study conducted in Angus cattle reported that EFE supplementation modified ruminal protozoal populations, notably through a reduction in *Diplodinium* protozoa and increased ruminal disappearance rates without altered ruminal papillae morphology or animal health [35].

Although the individual effects of SC and EFEs on ruminal fermentation and feed utilization have been extensively documented, their combined application under SSF conditions remains poorly explored, particularly for AHs. Limited evidence suggests that the integration of SC and EFEs during the short-term SSF (24 h) of alperujo improves ruminal digestibility, metabolizable energy availability, and TVFA production compared with single treatments [35]. However, no study to date has evaluated this integrated biotechnological strategy over a longer fermentation period or for the valorization of AHs, nor its potential impact on ruminal greenhouse gas emissions.

Therefore, the present study aimed to investigate, for the first time, the combined application of SC and EFEs under long-term SSF conditions (14 d) to valorize AHs. Specifically, we evaluated the effects of this approach on chemical composition, ruminal fermentation kinetics, microbial populations, nutrient degradability, and methane and carbon dioxide emissions, in comparison with individual treatments. This work seeks to contribute to the development of sustainable fermentation-based feed bioprocesses and to support circular bioeconomy strategies in ruminant nutrition.

2. Materials and Methods

2.1. Almond Hull Collection and Processing

Fresh AHs were collected during the summer from six industrial almond-processing plants located in the Sfax region (Tunisia). Sampling was conducted weekly over five consecutive weeks. During each sampling event, AHs were manually cleaned to remove foreign materials and impurities, pooled in equal proportions across processing plants, thoroughly homogenized, and considered as a single composite sample per week. The pooled samples were oven-dried at 55 °C for 48 h, ground to 1 mm using a Retsch mill, and sterilized by autoclaving at 121 °C for 30 min to eliminate native microorganisms. After cooling, sterilized samples were stored in sterile containers.

2.2. Experimental Treatments

AHs collected each week was assigned to four experimental treatments. For the SC treatment, 100 g DM of AHs were moistened to a final moisture content of 67.8% using sterile distilled water and inoculated with 100 mg DM of SC (Yea-Sacc® 1026; All-

tech Inc., Lexington, KY, USA), providing 5×10^{10} CFU g^{-1} DM. For the EFE treatment, 100 g DM of AHs were similarly moistened and supplemented with 100 µL of a commercial EFE preparation (Cellulase plus and Xylanase plus (1:1/*V*:*V*); Dyadic International Inc., Jupiter, FL, USA), produced by *Trichoderma longibrachiatum* and characterized by activities of 2267 U mL^{-1} xylanase, 1161 U mL^{-1} endoglucanase, and 113 U mL^{-1} exoglucanase. In the combined treatment (SC + EFEs), 100 g DM of AHs were moistened to 67.8% and simultaneously supplemented with both SC (100 mg DM) and EFEs (100 µL). The control treatment consisted of 100 g DM of AHs moistened to 67.8% with sterile distilled water without additives. All treatments were incubated under aerobic SSF conditions at 40 °C for 14 d. Moisture content and incubation conditions for the SC treatment were based on those of Sechrist [36] which ensure optimal SC development in AHs, whereas the EFE dosage was selected according to the work of Abid et al. [20], which achieves maximal EFE efficacy in Ahs, and the SC dosage was determined according the industrial recommendation. Each treatment was prepared in three technical replicates for each of the five weekly biological replicates [37]. The technical replicates corresponding to each treatment within each week were pooled, oven-dried at 55 °C for 48 h, ground to pass through a 1 mm sieve using a Retsch mill and stored in sterile containers until analysis.

2.3. Chemical Analysis

All chemical analyses were performed in triplicate (technical replicates) for each biological replicate, and mean values were used for statistical analysis. DM was determined according to AOAC method 934.01 by oven-drying samples at 105 °C for 3 h. CP was quantified using the Kjeldahl procedure (AOAC 978.04), with nitrogen content multiplied by 6.25 to estimate the CP content. Ether extract (EE) was determined by Soxhlet extraction using petroleum ether as the solvent (AOAC 920.39). Ash content was measured by incineration in a muffle furnace at 550 °C for 6 h (AOAC 942.05) [38]. Cell wall components, including NDF, acid detergent fiber (ADF), and acid detergent lignin (ADL), were analyzed using an ANKOM 200 fiber analyzer (ANKOM Technology, Macedon, NY, USA) following the procedures described by Van Soest [39]. Non-fiber carbohydrates (NFCs) were calculated according to the NRC equation [40]:

$$\mathrm{NFC} = 1000 - (\mathrm{NDF} + \mathrm{CP} + \mathrm{EE} + \mathrm{ash}) \quad (1)$$

where all components are expressed on mg g^{-1} DM.

2.4. Rumen Fluid Collection and In Vitro Fermentation

Rumen fluid was sampled weekly over a period of five consecutive weeks from three clinically healthy adult dairy goats (3 years old; mean body weight 60 ± 2.1 kg) at a commercial abattoir in Tunis, Tunisia. For one month prior to slaughter, the animals were maintained on a uniform basal diet comprising 0.5 kg DM oat hay and 0.5 kg DM commercial concentrate per day and with ad libitum access to fresh water. Immediately following slaughter, the rumen contents were collected into pre-warmed (39 °C) insulated thermos flasks flushed with CO_2. Samples were transported to the laboratory within 10 min. Upon arrival, the rumen contents from each goat were filtered through four layers of cheesecloth under continuous CO_2 flushing (50 mL min^{-1}) using a sterile gas dispersion tube connected to a CO_2 cylinder, while maintaining the temperature at 39 °C. The filtered rumen fluid from all three goats was combined in equal proportions to form a homogeneous inoculum, which was treated as a single biological replicate.

A buffer solution was prepared according to Menke and Steingass [41] and continuously flushing with CO_2 flow (50 mL min^{-1}) at 39 °C for approximately 30 min prior to inoculation to maintain strict anaerobic conditions [42]. The buffer was then mixed

with rumen fluid at a 2:1 (buffer/rumen fluid, v/v) ratio under continuous CO_2 flow (50 mL min^{-1}) at 39 °C in a shaking water bath (300 rpm). The pH of the buffered rumen inoculum was adjusted to 6.8 ± 0.05 before incubation.

In vitro ruminal fermentation was carried out under anaerobic conditions following the semi-automated gas production protocol of Theodorou et al. [43]. For each experimental run, 200 mg DM of each treated AH sample was weighed into 120 mL amber serum bottles, which were then filled with 30 mL of the pre-warmed, buffered rumen inoculum, corresponding to a substrate-to-inoculum ratio of 6.7 mg DM mL^{-1}. Each treatment within a biological replicate was prepared in three technical replicates (bottles), and three blank bottles containing inoculum only were included in each run.

Immediately, bottles were purged with CO_2 to eliminate any remaining oxygen then immediately sealed with butyl rubber stoppers and aluminum crimps. The sealed bottles were incubated at 39 °C in a shaking water bath operating at 120 rpm to ensure uniform mixing. Time zero of each bottle was defined as the moment that each bottle was placed in the shaking water bath. All fermentation bottles were inoculated within 30 min of rumen fluid collection. Serum bottles were randomly positioned within the shaking water bath and repositioned after each gas measurement to minimize positional effects.

Headspace gas pressure was recorded at 2, 4, 6, 8, 12, 24, 48, 72, and 96 h using a pressure transducer (PX4200-0100GI, Omega Engineering, Laval, QC, Canada) connected to a data logger (Data Tracker 200, Data Track Process Instruments Ltd., Christchurch, New Zealand). The pressure transducer was calibrated prior to each incubation run according to the manufacturer's instructions. After each measurement, gas pressure was fully released using a 23-gauge needle to prevent inhibition of fermentation due to excessive pressure: Gas samples were collected in gas-tight sampling bags for subsequent gas composition analysis. All fermentation procedures were performed using sterile materials pre-warmed to 39 °C prior to inoculation.

Gas volume (G_V, mL) was calculated as follows:

$$G_V(t) = \frac{G_P(t) \times \left(V_f - V_i\right)}{P_{atm}} \quad (2)$$

where G_P is recorded gas pressure (bar), V_f is bottle volume (mL), V_i is inoculum volume (mL), and P_{atm} is atmospheric pressure (bar).

Cumulative gas production data were fitted to the nonlinear model of France et al. [44] to estimate kinetic parameters:

$$Y_t = \mathrm{A} \times \left(1 - e^{(-C \times (t - Lag))}\right) \quad (3)$$

where Y is cumulative gas production (mL g^{-1} DM), A is asymptotic gas production, C is the fractional rate of gas production (%/h^{-1}), Lag is lag time (h), and t is incubation time (h).

Five incubation runs were performed, corresponding to the five biological replicates. Mean values of technical replicates were used for subsequent statistical analyses.

2.5. Fermentation End Products, Microbial Counts, and Degradability

At the end of incubation (96 h, gas production curves reached a plateau before the end of incubation, indicating completion of fermentative activity), fermentation was terminated by placing the bottles in an ice-water bath for 30 min [42]. Rumen fluid pH was measured immediately using a portable pH meter (Orion Star A221, Thermo Scientific, Montreal, QC, Canada). Then, a 0.2 mL aliquot of rumen fluid was mixed with 1.8 mL of 10% formalin–saline solution and stored at room temperature. The suspension was thoroughly homogenized, and a 1 mL subsample was loaded into a Levy–Sedgewick–Rafter counting

chamber (S52 glass; Pyser-SGI, Edenbridge, Kent, UK) for the enumeration of protozoa using a light microscope at 100× magnification according to Galyean [45]. Another aliquot of 1 mL was immediately loaded into a Petroff–Hausser counting chamber (Hausser Scientific®, Horsham, PA, USA) and examined under oil immersion at 1000× magnification according to Galyean [45]. Fermentation residues were filtered through Whatman No. 541 filter paper (Whatman Scientific Ltd., Maidstone, Kent, UK). A 5 mL aliquot was centrifuged at 3000 r·min^{-1} for 10 min, after which the clarified supernatant was collected, acidified with 1 N H_2SO_4 at a 5:2 (v/v) ratio, stored at −20 °C, and subsequently analyzed for ammonia nitrogen (NH_3–N) using the micro-Kjeldahl method [38]. Another 2 mL aliquot of the supernatant was centrifuged at 4000× g for 15 min at 4 °C. The resulting supernatants were mixed with 0.2 mL of a meta-phosphoric acid solution (250 g/L) at 4 °C for 30 min, the mixtures were centrifuged at 10,000× g for 10 min at 4 °C, and subsequently, the supernatants were collected for VFA analysis by gas chromatography (Shimadzu GC-2014, Tokyo, Japan) [42,46]. Fermentation residues were analyzed for DM [38] and NDF [39] in order to calculate the DMD and neutral detergent fiber degradability (NDFD). Degradability values were expressed as the proportion of material degraded relative to the initial amounts, corrected for residual DM and NDF measured in blank incubations. Gas samples collected in sampling bags were analyzed for CH_4 and CO_2 concentrations using a portable gas analyzer (Dräger X-am 8000, Drägerwerk AG & Co. KGaA, Haan, Germany) equipped with independent infrared sensors and a sampling pump, as previously applied in an in vitro ruminal gas composition analysis [47]. This setup allowed simultaneous measurement of CH_4 and CO_2 without cross-interference. The instrument was factory calibrated prior to use. Methane and carbon dioxide production was corrected for gas generated in blank incubations. Gas outputs were quantified using multiple complementary metrics to provide a comprehensive assessment of fermentation performance, as previously demonstrated in an in vitro ruminal gas composition analysis [21]. Absolute gas production per unit of dry matter (mL/g DM) was calculated to determine the total emissions relative to substrate input. Gas production per unit of dry matter degradability (mL/g DMD) normalized emissions to substrate degradability, allowing accurate comparison across treatments with variable fermentability. In addition, CH_4 and CO_2 were expressed as a proportion of the total gas production (%) to evaluate shifts in fermentation pathways independently of total gas volume.

2.6. Statical Analysis

Gas production kinetics were estimated using the nonlinear regression procedure of SAS (version 9.1; SAS Institute Inc., Cary, NC, USA). Other data were analyzed by an analysis of variance (ANOVA) using a completely randomized design, with the treatment as a fixed effect and biological replicate as the experimental unit, and according to the following model

$$Y_{ij} = \mu + T_i + \varepsilon_{ij} \quad (4)$$

where Y_{ij} is the dependent variable, μ is the overall mean, T_i is the fixed effect of treatment, and ε_{ij} is the residual error. When the treatment was significative, means were compared using Tukey's multiple range test, and differences were considered significant at $p \leq 0.05$. Prior to analysis, the assumptions of normality and homogeneity of variances were verified. Normality of the model residuals was evaluated using the Shapiro–Wilk test ($p > 0.05$) and homogeneity of variance was evaluated using Levene's test ($p > 0.05$).

3. Results

3.1. Effects of Saccharomyces cerevisiae and Exogenous Fibrolytic Enzymes on the Chemical Composition

SC, EFE, and SC + EFE treatments significantly modified the chemical composition of AHs (Table 1). Compared with the control, CP content increased significantly following SC treatment and was further enhanced by the combined SC + EFE treatment, whereas EFEs alone had no effect. EE content also increased significantly with SC and reached the highest value with SC + EFEs. In contrast, EFE treatment did not affect EE content. NDF and ADF concentrations were significantly reduced by EFE and SC + EFE treatments, whereas SC alone did not affect the fiber fractions. The ADL content remained unchanged across all treatments. Ash concentration increased with SC-containing treatments, while EFEs alone had no effect. The NFC concentration was improved by EFE treatment and decreased by SC and SC + EFEs.

Table 1. Effects of *Saccharomyces cerevisiae* and exogenous fibrolytic enzymes on the chemical composition of almond hulls.

Item	Control	SC	EFEs	SC + EFEs	SEM	*p*-Value
Crude protein (g/kg dry matter)	69 ^{c}	92 ^{b}	68 ^{c}	133 ^{a}	5.1	***
Ether extract (g/kg dry matter)	31 ^{a}	39 ^{b}	30 ^{a}	46 ^{c}	0.9	***
Neutral detergent fiber (g/kg dry matter)	331 ^{a}	336 ^{a}	308 ^{b}	316 ^{b}	7.4	**
Acid detergent fiber (g/kg dry matter)	200 ^{a}	203 ^{a}	181 ^{b}	178 ^{b}	3.1	**
Acid detergent lignin (g/kg dry matter)	94	93	91	94	2.3	NS
Ash (g/kg dry matter)	73 ^{b}	88 ^{a}	75 ^{b}	95 ^{a}	4.8	*
Non-fiber carbohydrates (g/kg dry matter)	496 ^{b}	445 ^{c}	519 ^{a}	410 ^{d}	8.7	***

^{a,b,c,d} Means within the same row with different superscripts differ significantly ($p < 0.05$); *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS: not significant ($p \geq 0.05$); SEM: standard error of the mean. SC: *Saccharomyces cerevisiae*; EFEs: exogenous fibrolytic enzymes.

3.2. Effects of Saccharomyces cerevisiae and Fibrolytic Enzymes on Ruminal Fermentation

The effects of SC, EFE, and SC + EFE treatments on in vitro ruminal fermentation, nutrient degradability, and greenhouse gas emissions of AHs are summarized in Table 2. All treatments modulated gas production kinetics through distinct mechanisms. EFEs increased the fractional rate of gas production and reduced the lag phase, whereas SC enhanced asymptotic gas production. The combined SC + EFE treatment reduced the lag phase and increased asymptotic gas production relative to the control. Ruminal pH and NH_3–N concentrations were unaffected by all treatments. TVFA increased with SC and by SC + EFEs, accompanied by a reduction in the acetate-to-propionate ratio, while EFEs alone had no effect. Bacterial populations increased with SC and with SC + EFEs. In contrast, protozoal populations decreased with SC + EFEs. DMD and NDFD were improved by SC and further increased under SC + EFEs. CH_4 and CO_2 production per unit of DM did not differ among treatments; however, when expressed per unit of DMD or as a proportion of the total gas, CH_4 and CO_2 emissions were significantly reduced under SC + EFEs, respectively, compared with the control.

Table 2. Effects of *Saccharomyces cerevisiae* and exogenous fibrolytic enzymes on ruminal fermentation of almond hulls.

Item	Control	SC	EFEs	SC + EFEs	SEM	*p*-Value
Gas kinetics						
A (mL/g dry matter)	210 ^{c}	238 ^{b}	222 ^{bc}	256 ^{a}	8.2	**
C (%/h)	3.44 ^{b}	3.46 ^{b}	3.62 ^{a}	3.43 ^{b}	0.181	*
Lag (h)	0.48 ^{a}	0.54 ^{a}	0.15 ^{b}	0.14 ^{b}	0.107	**
Fermentation						
pH	6.18	6.20	6.18	6.19	0.031	NS
NH_3–N (mg/L)	121	124	122	126	2.7	NS
TVFA (mmol/g dry matter)	84.6 ^{c}	86.7 ^{c}	92.7 ^{b}	100.2 ^{a}	4.31	***
Ac (% TVFA)	62.2 ^{a}	61.8 ^{ab}	62.5 ^{a}	60.5 ^{b}	1.92	*
Pr (% TVFA)	25.2 ^{b}	26.6 ^{ab}	24.9 ^{b}	27.3 ^{a}	0.93	*
Bu (% TVFA)	9.2	9.4	9.1	9.3	0.61	NS
Ac/Pr	2.46 ^{a}	2.32 ^{ab}	2.51 ^{a}	2.21 ^{b}	0.08	*
Degradability						
DMD (g/kg)	471 ^{c}	526 ^{b}	496 ^{c}	604 ^{a}	8.2	**
NDFD (g/kg)	401 ^{c}	429 ^{b}	413 ^{bc}	463 ^{a}	7.3	**
Microbiota						
Bacteria ($\times 10^8$ cells/mL)	9.3 ^{c}	12.2 ^{b}	9.9 ^{c}	14.1 ^{a}	1.12	**
Protozoa ($\times 10^5$ cells/mL)	3.39 ^{a}	3.12 ^{ab}	3.33 ^{a}	2.93 ^{b}	0.160	*
greenhouse gas						
CH_4/DM (mL/g dry matter)	22.3	22.4	22.8	23.3	2.11	NS
CH_4/total gas (% total gas)	10.6 ^{a}	9.6 ^{ab}	10.2 ^{a}	9.1 ^{b}	0.84	*
CH_4/DMD (mL/g degraded dry matter)	47.3 ^{a}	42.6 ^{ab}	45.9 ^{a}	38.6 ^{b}	4.26	*
CO_2/DM (mL/g dry matter)	62.3	61.9	63.3	64.4	3.61	NS
CO_2/total gas (% total gas)	29.7 ^{a}	26.8 ^{ab}	28.5 ^{a}	25.1 ^{b}	1.81	*
CO_2/DMD (mL/g degraded dry matter)	132.2 ^{a}	119.6 ^{ab}	127.6 ^{a}	106.6 ^{c}	7.22	*

^{a,b,c} Values with different superscripts within the same row are significantly different ($p < 0.05$); *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS, not significant ($p \geq 0.05$); SEM: standard error of the mean; A: asymptotic gas production; C: fractional rate of gas production; Lag: lag phase before fermentation; TVFA: total volatile fatty acids; Ac: acetate; Pr: propionate; Bu: butyrate; Ac/Pr: acetate-to-propionate ratio; NH_3–N: ammonia nitrogen; DMD: dry matter degradability; NDFD: neutral detergent fiber degradability; Bacteria: rumen bacteria; Protozoa: rumen protozoa; CH_4/DM: methane per unit of dry matter; CH_4/DMD: methane produced per unit of degraded dry matter; CH_4/total gas: proportion of methane per total gas production; CO_2/DM: carbon dioxide per unit of dry matter; CO_2/DMD: carbon dioxide produced per unit of degraded dry matter; CO_2/total gas: proportion of carbon dioxide per total gas production; SC: *Saccharomyces cerevisiae*; EFEs: exogenous fibrolytic enzymes.

4. Discussion

The chemical composition of untreated AHs used in this study, collected from Tunisia, was characterized by a high NFC content (496 g/kg DM), low CP content (69 g/kg DM), and moderate NDF content (331 g/kg DM). Comparable compositional profiles have been reported for AHs from other geographic regions, including Spain [10,48], Iran [18] and Palestine [21]. These characteristics inherently limit the nutritive value of AHs for ruminants, as the imbalance between rapidly fermentable carbohydrates and insufficient nitrogen availability constrains microbial protein synthesis and fibrolytic activity, resulting in suboptimal fiber degradation and reduced overall feed efficiency [49,50]. In vitro ruminal digestibility assays, based on gas production and fermentation kinetics and widely used to evaluate feed nutritional value [41,43], showed that goats had a limited ability to degrade NDF and ADF in AHs, with a DMD of 47.1%, NDFD of 40.1%, and relatively low total volatile fatty acid (TVFA) production (84.6 mmol/g DM). Although no studies have specifically assessed AH degradability in goats, these results are comparable to the moderate ruminal degradability of untreated AHs reported in sheep, with DMD ranging from 49.9 to 60.5%, NDFD from 43.3 to 50.3%, and TVFA from 52 to 75 mmol/g DM [18,19,21,48]. These

findings underscore the intrinsic limitations of AHs as a ruminant feed and highlight the need for targeted interventions to enhance degradability, optimize ruminal fermentation, and improve overall nutrient utilization.

4.1. Effects of Exogenous Fibrolytic Enzymes Treatment

Treatment of AHs with EFEs selectively decreased the NDF and ADF contents while increasing NFCs. These findings are consistent with previous studies showing that EFE treatment of pure cellulose and xylan increased reducing sugar concentrations, including glucose and xylose [51,52] Similar effects have been reported for conventional feeds such as bermudagrass haylage, where EFE treatment increased water-soluble carbohydrates, glucose, and xylose while reducing NDF content [51,53], as well as for alternative feeds such as peanut hulls, in which the NFC content increased alongside reductions in ADF and NDF contents [54]. The reduction in structural carbohydrates following EFE treatment enhanced early-phase ruminal fermentation by shortening the lag time and increasing the rate of gas production, as the NFC content serves as a readily fermentable source and chemoattractant for microbial colonization [55]. In agreement with this interpretation, previous studies have reported that treating corn silage and alfalfa hay with EFEs produced by *Trichoderma longibrachiatum* stimulated ruminal bacterial attachment, thereby enhancing fermentation rates [56]. Accelerated fermentation during the initial phase may further increase rumen turnover and voluntary feed intake, contributing to improved feed utilization efficiency [57,58]. However, EFE treatment did not affect asymptotic gas production or total fermentation extent, which aligns with the results of previous studies on tropical forages treated with EFEs, where improvements in VFA production were observed after short incubation times (5 and 10 h) but not longer incubation periods (24 h) in lambs [59]. Similarly, an in vivo study in lambs demonstrated that EFE supplementation increased feed intake by promoting a longer feeding time and increased chewing activity without affecting nutrient degradability or growth performance [60]. This limited late-phase effect likely results from the proteolytic degradation of EFEs and/or substrate saturation by endogenous rumen enzymes [56,61].

4.2. Effects of Saccharomyces cerevisiae Treatment

The SSF of AHs with SC significantly altered their chemical composition by increasing CP, EE, and ash contents, while reducing NFCs. These compositional shifts are in line with previous studies showing that SC growth during SSF produces extracellular enzymes capable of altering the substrate composition [62]. The reduction in NFCs is attributed to the catabolism of carbohydrates by SC to sustain biomass production and energy metabolism [63]. Similar effects have been reported in soybean meal treated with SC under SSF, which led to higher CP and ash contents and reduced NFCs [26], as well as in yellow wine wastes, where CP content progressively increased throughout the fermentation period [64]. Additionally, SSF with another yeast (*Saccharomyces boulardii*) enhanced the CP and ash contents in rice husks, although a slight decline in EE was observed [63]. Collectively, these results from both conventional and non-conventional feed substrates highlight that SSF with yeast consistently improves the nutritional profile of diverse agro-industrial by-products. The enhanced CP content in AHs exceeded the minimum level required ($\geq$70 g/kg DM) to sustain optimal ruminal fibrolytic activity [49,50], indicating that SSF with SC could help alleviate nitrogen limitation in the substrate and thereby promote the growth of fibrolytic microorganisms. This observation is consistent with in vitro ruminal fermentation results, which demonstrated the enhanced activity of rumen bacteria. Previous in vivo studies have demonstrated that supplementation with SC-fermented products promotes the proliferation of fibrolytic bacteria such as *Ruminococcus flavefaciens*

and *Fibrobacter succinogenes* in cattle [65] and improves both the diversity and fibrolytic activity of the rumen microbiome in sheep fed yeast-enriched diets [66]. The increase in EE content in AHs can be attributed to lipid accumulation within the SC biomass during fermentation [62,67], although an elevated EE content in ruminant diets can inhibit fiber-digesting bacteria when exceeding ~6% DM [68]. The levels observed here remained below the inhibitory thresholds, suggesting no adverse effects on ruminal fibrolytic populations. These findings underscore the need to consider the effects of SSF-induced changes in EE when evaluating the substrate nutritive value. The rise in ash content resulted from organic matter loss and mineral enrichment from the yeast biomass [62,63]. While such enrichment may enhance mineral availability in ruminant diets, further analysis is required to identify the specific minerals affected and assess their nutritional significance.

Collectively, these compositional modifications translated into enhancing DMD, NDFD, and overall fermentation. Previous studies have shown that increases in NDFD are positively associated with a higher voluntary feed intake (≈0.17 kg per unit NDFD) and enhanced milk yield (≈0.25 kg fat-corrected milk per unit NDFD) in lactating ruminants [69]. In vivo studies support these links; dairy cows fed SC exhibited increased DMD, NDFD, and energy utilization, resulting in improved feed efficiency and milk production [70], while sheep supplemented with SC demonstrated similar enhancements in NDFD and growth performance [66]. VFA production increased without significant shifts of molar proportions, consistent with the results of in vivo studies of low-quality forage supplemented with SC [65,70]. These results indicate that SSF can intensify ruminal fermentation without disrupting core fermentation pathways. Additionally, NH_3–N concentrations remained stable and within the optimal range for bacterial growth and microbial activity (50–250 mg/L) [71], despite the increased CP content, suggesting efficient microbial protein synthesis, as previously reported for low-quality feeds enriched with SC [65]. Ruminal pH was maintained within the physiological range, supporting metabolic homeostasis in the rumen despite changes in substrate fermentability and degradability [72]. This stability may be attributed to the capacity of SC to prevent ruminal acidosis [73].

Despite these positive effects on fermentation and digestibility, greenhouse gas emissions (CH_4 and CO_2) were not significantly affected. This observation aligns with meta-analytical evidence indicating that live yeast supplementation typically has minimal or negligible effects on enteric methane emissions [74].

4.3. Effects of Combining Saccharomyces cerevisiae and Fibrolytic Enzymes Treatment

The combined SC + EFE treatment of AHs under SSF produced synergistic improvements after 14 days, exceeding the effects of individual treatments. Specifically, the CP and EE contents further increased, while the NFC content further decreased and structural carbohydrates decreased as with the EFE treatment. These compositional changes indicate improved substrate accessibility by SC supports growth and metabolic activity during SSF. In contrast, a short SSF (1-day) with SC + EFEs applied to alperujo did not produce synergistic increases in CP or EE and resulted in a greater reduction in NDF compared with individual treatments [35]. These discrepancies underscore the importance of SSF duration and substrate characteristics in determining the effectiveness of a combined bioprocessing strategy. The extended fermentation period likely allowed synergistic interactions between enzymatic fiber depolymerization and yeast metabolism to fully develop.

This modification of the chemical composition of AHs leads to changes in the rumen microbial ecosystem. Rumen bacterial populations further increased, while rumen protozoal populations decreased, likely due to a combination of fat-mediated protozoal suppression [68] and reduced predation on bacterial cells by protozoa [75,76]. By contrast, the short period of SSF of SC + EFE treatment of alperujo did not stimulate bacterial growth

and instead increased protozoal abundance relative to single treatments [35], again emphasizing the influence of substrate characteristics and processing conditions on ruminal microbial dynamics. This shift in the ruminal microbial community resulted in enhanced fermentation kinetics, with higher asymptotic gas production, a reduced lag time, and improved DMD and NDFD and VFA production, and shifted towards propionate production. This shift in VFA production can enhance energy efficiency and potentially improve milk production [65,77].

The combined SC + EFE treatment also reduced CH_4 and CO_2 emissions relative to substrate utilization and fermentation pathways, highlighting its potential for environmentally sustainable feed processing. This mitigation may be the result of protozoal suppression, which could indirectly diminish the activity of methanogenic microbes [77,78], and increased lipid availability, which may promote biohydrogenation pathways that compete with methane formation [79]. Confirmation of these microbial processes will require the application of advanced molecular techniques and metagenomic analyses to elucidate the specific microbial interactions and pathways underlying greenhouse gas mitigation. In addition, future in vivo studies will be essential to validate these findings. Despite changes in ruminal fermentation, chemical composition and ruminal microbial ecology, ruminal pH remained stable within the optimal range for microbial activity [72]. Similar pH stability was observed in the short-period of SSF with the SC + EFE treatment of alperujo [35], likely due to the ability of SC to buffer the rumen environment and reduce the risk of acidosis [73]. By simultaneously enhancing the nutrient value and suppressing greenhouse gas production, this combined treatment strategy offers a dual benefit: optimizing feed efficiency while reducing the ecological footprint of ruminant production systems. However, the reduction in NFCs associated with SC application may affect the energy balance of ruminant diets if not carefully formulated.

5. Conclusions

The biotechnological valorization of AHs via SSF using EFEs and SC effectively upgrades low-value by-products into high-quality ruminant feed. This combined strategy improves CP, ash and EE contents, reduces fiber content, enhances ruminal degradability and bacterial populations, and lowers CH_4 and CO_2 emissions relative to substrate utilization and fermentation pathways. The synergistic effects highlight the value of integrating enzymatic depolymerization with yeast metabolism in SSF design. However, the reduction in NFCs associated with SC may affect the feed energy content and should be considered in diet formulation. The process is technologically simple, requires minimal water, and relies on commercially available inputs, supporting industrial feasibility. Future research should address pilot-scale validation, optimization under variable conditions, techno-economic assessment, and in vivo confirmation of animal performance and environmental benefits. Advanced molecular techniques will also be needed to characterize the ruminal microbiota and elucidate the mechanisms underlying greenhouse gas mitigation.

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Abbreviations

The following abbreviations are used in this manuscript:

A	Asymptotic gas production
Ac	Acetate
ADL	Acid detergent lignin
AHs	Almond hulls
Bu	Butyrate
C	Fractional rate of gas production
CP	Crude protein
DM	Dry matter
DMD	Dry matter degradability
EE	Ether extract
EFE	Exogenous fibrolytic enzyme
GP	Gas pressure
Gv	Gas volume
Lag	Time at which gas production starts
NDF	Neutral detergent fiber
NDFD	Neutral detergent fiber degradability
NFC	Non-fiber carbohydrate
NS	Not significant
Patm	Atmospheric pressure
Pr	Propionate
SC	*Saccharomyces cerevisiae*
SEM	Standard error of the mean
SSF	Solid-state fermentation
Vf	Bottle volume
VFA	Volatile fatty acid
Vi	Inoculum volume

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