Research article

Investigating variability of craft microbreweries spent grains for classification and incorporation into precision diet formulation through multivariate analyses

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ABSTRACT

Alternative feedstuffs offer a cost-effective and sustainable option for livestock nutrition, playing a crucial role in niche market development. Brewer's spent grains (BSG), a byproduct of the expanding craft microbrewery industry, are a particularly promising feed source due to their availability and nutrient content. However, variability in BSG composition poses challenges for their effective incorporation into precision diet formulations. This study aimed to evaluate the variability in the nutrient composition of BSG from craft microbreweries and classify them for precision diet formulation using multivariate analyses. BSG samples from 29 craft microbreweries were collected and analysed for their nutrient composition using wet chemistry methods. Principal components analysed included crude protein (CP), ash and protein corrected neutral detergent fiber (apNDFom), non-fibrous carbohydrates (NFC), and ether extract (EE). Principal component analysis (PCA) was employed to identify the most significant nutrient variations, and hierarchical clustering of the principal components was used to group the samples into four distinct clusters. These clusters were further evaluated through *in vitro* fermentation tests, assessing gas production, digestibility, and fermentation characteristics. Statistical analyses were conducted using R software. The principal components (energy (PC1) and protein (PC2)) were the primary factors driving BSG variability. Hierarchical clustering produced four distinct feed

clusters, which showed significant differences ($P < 0.05$) in fermentation profiles, The apNDFom digestibility varied across clusters, with energy-dense feeds (higher and lower energy grains) demonstrating higher digestibility ($P < 0.05$). The third cluster (CL3), characterized by low protein content, had significantly lower NH_3-N concentrations after fermentation ($P < 0.05$). Regarding gas and volatile fatty acids (VFA) production, clusters exhibited significant differences ($P < 0.05$) compared to an alfalfa standard, highlighting the diverse fermentation characteristics of BSG. The variability in energy and protein content among BSG samples results in distinct fermentation profiles, which can influence animal performance and environmental outcomes. These findings emphasize the importance of classifying BSG and incorporating precision formulation to mitigate adverse effects and maximize the benefits of this alternative feedstuff.

1. Introduction

Artisanal microbreweries are an international, high-growth sector with a constant increasing influx of new, small, and specialist ventures [1]. The global expansion of artisanal microbreweries has led to a substantial increase in the production of Brewer's spent grains (BSG), which constitutes approximately 85 % of total brewing resides [2] Between 2022 and 2023, the count of operational craft breweries saw a notable increase, hitting a record-breaking 9552 establishments. This figure encompasses various categories, including 2035 microbreweries, 3418 brewpubs, 3838 taproom breweries, and 261 regional craft breweries, producing approximately 8.7 million tons of coproduct waste per year [3]. The adoption of reuse and recycling practices plays a crucial role in converting waste into valuable resources [4]. Now more than ever, there's a pressing need to explore sustainable strategies in overlooked sectors such as small brewery value chains. This research aims to enhance understanding and facilitate better decision-making regarding waste disposal or valorisation [5]. Brewer's spent grains are recognized for their rich fiber, protein, and phenolic compounds and for use as human and animal feed supplements [6,7]. Annually, over 30 million tons of BSG are generated globally [2]. Given that BSG are generally considered waste from beer production, they are usually obtained for free or as a cheap feed and thus represent a unique opportunity for livestock producers to reduce input costs associated with feeding [8].

Despite its nutritional value, the utilization of BSG in livestock feed is limited by its high moisture content (up to 80 %), leading to rapid microbial spoilage and reduced shelf life [9]. This perishability poses challenges in storage and transportation, often resulting in BSG being underutilized or discarded, thereby contributing to environmental waste [9]. Though drying methods, ensiling, chemical treatments, and direct-feeding wet BSG have been explored [9], some of these methods are cost-prohibitive and may alter nutritional profile of BSGs further presenting inherent variations in BSG composition which complicates its standardization as a feed ingredient [10]. Additional environmental concerns could arise through nitrogen leaching in urine and faeces from mischaracterization of nutrient profiles [11]. Environmental concerns also arise from the improper disposal of BSG, which can lead to increased greenhouse gas emissions and other ecological impacts associated with leaking of nutrients into water sources [12]. The concept of upcycling BSG into value-added products aligns with sustainable practices and circular economy principles, yet its application in animal nutrition requires further investigation to ensure safety and effectiveness [7].

Generally, feedstuffs are identified according to their international feed number which consists of five digits assigned according to the chemical and biological data describing the feed [13]. The current classification for BSG classifies them as a protein feed. Per their classification, protein supplements are those with 20 % or more crude protein (CP) content and less than 18 % crude fiber [13]. However, there are other nutritional characteristics that may be overlooked if BSG are solely considered protein supplements. Additional complications arise when we consider the ability to utilize BSG for monogastric livestock species, however, some processing technologies are expanding the application of these feeds for other livestock species [2].

Overall, the nutritive value of ruminant feed is defined by its chemical composition and digestion rate in the rumen [14]. Studies should investigate the chemical composition and fermentation traits when evaluating feedstuffs and their inclusion in livestock diets, and to the knowledge of the authors this work has not been completed for BSG. The chemical composition of brewer's spent grains (BSG) varies significantly due to differences in microbrewery grain processing and beer styles, leading to heterogeneous effects on ruminal fermentation parameters. These variations impact the potential classification and optimal dietary inclusion of BSG in livestock feeds. It is hypothesized that distinct nutritional clusters of BSG can be identified and that their inclusion in livestock diets must account for these differences to optimize nutrient utilization and minimize environmental impacts. The objectives investigated herein are to evaluate the chemical composition of BSG from multiple microbreweries, to determine the ruminal fermentation characteristics of BSG and their variation across clusters, to classify BSG into distinct nutritional clusters based on compositional data using multivariate analysis, and to assess implications of BSG variability for diet formulation while highlighting the potential environmental and metabolic disturbances that may occur if the nutrient and chemical composition of the feeds are overlooked.

2. Materials and methods

2.1. Brewer's spent grains

Brewer's grains were collected for a period of three years from microbreweries in Reno-Nevada, USA. In total, grains were collected from five microbreweries, totalling 29 beer varietals. In brief, microbreweries were visited weekly depending on brewing days for collection of fresh BSG. In total, at least 5 samples (50 kg wet basis) were collected per beer style. Spot fresh samples were collected and

immediately frozen ($-20\,^\circ$ C) until time of analyses. The remainder of the grains were air dried in a self-built, elevated drier lined with charcoal fiberglass screen for a period of 48–72 h with daily mixing which gave the pre-dry matter weight (DM*). Commercial names of the beers and breweries were omitted from the manuscript. The varietals collected resulted in a total of 7 lager-style beers and 22 ale-style beers; the beer types and their nutritional compositions are listed on Table 1.

2.2. Proximate analysis

All pre-frozen samples were air-dried in a forced draft oven (60 °C) and ground to pass a 1-mm screen in a Wiley mill (Model 4, Thomas scientific, Swedesboro, NJ, USA 08085) to analyze for dry matter (DM; method 934.01), ash (method 942.05) according to AOAC [15], organic matter calculated as 100 minus ash concentration, neutral detergent fiber (NDF) and acid detergent fiber exclusive of residual ash (ADFom) was analysed according to Van Soest et al. [16] and adapted for the Ankom200 Fiber Analyzer with inclusion of alpha amylase (Ankom Technology, Macedon, NY, USA). The NDF content corrected for ash and protein (apNDFom) was estimated according to Mertens et al. [17] and Licitra et al. [18]. Additionally, CP (method 2001.11), according to AOAC [19] and ether extract (EE; method 920.39), AOAC [15], non-fibrous carbohydrates (NFC) were calculated as: NFC (% DM) $= 100 -$ [CP + NDF + EE + ash], the total digestible nutrients (TDN) content was computed utilizing empirical equations reported on NASEM [20] with assumption of digestibility coefficients as described in [Eq. (1)]:

$$= TDN = CP_{\%DM} \times 0.78 + NFC_{\%DM} \times 0.95 + 2.25 \times [EE_{\%DM}*0.86] + apNDFom_{\%DM}*0.6. \qquad [\text{Eq.1}]$$

2.3. Multivariate clustering and feedstuff classification

To examine the chemical composition variation of the BSG a two-step agglomerative hierarchical cluster (HC) analysis on the principal components (PC; HCPC) was performed on the CP, EE, apNDFom, and NFC contents of the BSG [21,22]. A total of five treatments were explored, one alfalfa sample (laboratory standard), and four different BSG clusters.

Table 1
Proximate chemical composition for 29 spent microbreweries spent grain varietals.

Style	N		Analitical fraction[a], g/kg DM basis							
		DM*	DM	OM	apNDFom	ADFom	EE	CP	NFC	TDN
Ales microbreweries										
Blonde	1	243.2	950.3	960.3	346.0	154.5	112.1	197.6	304.6	868.0
Porter	2	237.2	966.0	962.3	363.2	205.5	90.8	207.7	300.5	841.2
Red Rye	4	244.5	959.5	959.3	402.9	189.3	106.3	189.3	260.8	842.8
Double IPA	5	248.2	963.9	959.4	293.7	166.3	92.0	187.6	386.2	867.3
Stout	6	244.5	946.7	960.1	296.8	170.7	105.3	178.6	379.4	881.5
Pale Ale	7	268.5	959.8	970.0	301.1	144.8	92.3	218.6	358.1	870.0
Hazy IPA	8	254.2	952.2	956.4	314.0	167.9	111.6	181.2	349.5	877.8
Chile	12	253.8	953.5	963.5	297.4	135.0	94.1	163.2	408.9	876.3
IPA	13	254.6	945.4	966.7	236.0	122.1	96.5	160.2	474.0	903.6
Stout	14	255.8	955.5	967.9	209.9	147.5	98.6	197.6	461.9	909.6
Kolsen	15	268.2	943.3	962.3	323.6	148.6	110.7	179.7	348.3	879.3
Red Rye	16	249.3	959.4	968.0	374.7	185.1	95.6	212.1	285.7	846.6
Hazy IPA	17	248.3	937.2	968.6	305.0	149.3	125.3	223.3	315.1	898.9
Pale Ale	18	244.5	946.6	965.3	416.5	185.7	115.0	221.5	212.3	846.9
Kolsen	19	249.9	974.3	963.7	336.3	153.8	84.0	197.7	345.7	846.9
Double IPA	20	241.2	963.9	954.3	303.2	159.3	119.2	252.8	279.0	874.9
Hazy Pale Ale	21	245.3	954.6	956.5	418.2	140.5	133.6	223.8	180.8	855.8
Oat beer	22	255.6	965.6	970.8	432.2	199.1	96.0	135.3	307.3	842.6
Pumpkin Ale	25	245.2	957.0	965.0	340.2	124.8	93.4	150.1	381.3	864.2
Cream Ale	26	248.9	969.7	967.6	436.0	146.2	107.9	241.5	182.2	831.8
Amber Ale	27	281.1	947.2	962.6	383.5	126.9	126.6	130.2	322.3	882.9
Stout	28	214.7	953.5	968.3	428.0	163.8	122.8	210.2	207.4	855.3
Lagers microbreweries										
Dark Lager	29	225.8	942.0	969.2	459.3	170.8	102.7	212.5	194.7	825.0
Oktoberfest	3	211.1	960.7	965.8	303.6	139.4	89.9	192.3	380.0	867.1
Mexican Lager	23	235.1	956.2	966.8	505.4	162.4	111.4	208.5	141.5	815.8
Lager	24	214.5	948.5	959.3	410.0	146.8	103.3	177.9	268.1	839.3
India Pale Lager	9	281.2	945.1	969.8	215.6	123.7	104.7	190.1	459.4	916.7
Oktoberfest	10	277.5	965.5	973.8	303.1	140.3	84.7	198.9	387.1	868.6
Pilsner	11	268.5	951.6	968.2	343.9	153.0	101.2	203.6	319.5	864.6

[2]29,3,23,24,9,10,11 are lager beers, all other beers are ales.

[a] DM* = dry matter from original air pre-drying, DM = dry matter, OM = organic matter, apNDFom = neutral detergent fiber exclusive of residual ash and protein, ADF = acid detergent fiber exclusive of residual ash, EE = ether extract, CP = crude protein, NFC = non-fibrous carbohydrates, TDN = total digestible nutrients, Double IPA$_a$ = double india pale ale beer, Double IPA$_b$ = hazy double india pale ale beer, Stout$_a$ = sweet milk stout, Stout$_b$ = imperial American, Stout$_c$ = oatmeal stout beer.

2.4. In vitro incubation, design, and sampling

Rumen fluid was collected from three rumen-cannulated crossbred Angus steers (650.2 ± 70.7 kg body weight, 11.7 ± 1 years old), fed *ad libitum* an alfalfa-based diet (DM: 924 g/kg; CP: 204 g/kg; NDF: 352 g/kg; TDN: 601 g/kg) for four weeks prior to sampling. Fluid was obtained after 12 h fasting, stored in pre-warmed thermal containers (39 °C), mixed uniformly, blended to separate microorganisms from particulates, and filtered through four layers of cheesecloth.

For incubation, 4 mL of rumen fluid was added to 150 mL sealed anaerobic Wheaton bottles containing 200 mg sample and 14 mL buffer solution [23] under continuous N_2 flushing. Incubations were conducted at 39 °C with pH adjusted to 6.8 using 1M HCl, and bottles were placed in an orbital shaker (90 rpm) for incubation periods of 3, 6, 9, 12, 24, 36, and 48 h. Five replicates per treatment and time point were analysed, alongside five replicates of an alfalfa standard for accuracy assessment. Blank bottles (buffer and inoculum only) were included to correct gas measurements.

Measurements included pH, gas pressure, and gas composition (CH_4 and CO_2). Samples for VFA, NH_3-N, and NDF degradability were collected and processed at designated time points. Microbial populations were not assessed but bottles used for pH and gas analysis were retained for consistency in gas production and composition.

2.5. Analysis

2.5.1. Gas pressure and analysis
Gas pressure was measured using a Druck DPI 104-IS pressure gauge. CH_4 and CO_2 concentrations were analysed using a Gow Mac thermal conductivity gas chromatograph equipped with a Porapak Q column (60 °C) and helium as the carrier gas. Quantifications were corrected for blank values and calculated using certified standard mixes (1 % H_2, 5 % CH_4, 94 % CO_2; Praxair Distribution, Inc.).

2.5.2. NH_3-N and VFA
Supernatants from centrifuged samples ($1500 \times g$, 10 min) were stored at −20 °C until analysis. NH_3-N was quantified spectrophotometrically at 625 nm (Biotek Synergy HT). VFAs were analysed on a gas chromatograph (Agilent Technologies 6890N) with a flame ionization detector and fused silica capillary column. Standard calibration curves were prepared using known VFA standards.

2.5.3. In vitro NDF digestibility
Following Van Soest et al. [24], samples were treated with α-amylase and NDF solution, autoclaved at 105 °C for 60 min, and filtered using pre-weighed Whatman #2 filter papers. Filters were rinsed sequentially with hot water (>90 °C), ethanol, and acetone, dried, and weighed to determine digestibility.

2.5.4. Gas production and fermentation dynamics
A two-pool Gompertz model with a single lag [25] was fitted to the cumulative gas production data. The model was optimized using nonlinear least squares (nls function in R), minimizing error via the "NL2SOL" algorithm [26,27].

2.6. Statistical analysis

In vitro data were analysed as a linear mixed model, following [Eq. (2)]:

$$[Eq.2] = Y_{ijk} = \beta_0 + \beta_j C_j + \beta_k T_k + \beta_{jk} C_j T_k + \eta + \eta_i R_i + \epsilon$$

where Y_{ijk} is the response for the ith observation, C_j is the feed cluster, T_k represents the incubation time in hours, and $C_j T_k$ represents their interaction. Random effects included the intercept (η) and run (R_i).

Pairwise comparisons of estimated marginal meals were performed using the emmeans package with Kenward-Roger degrees of freedom [28]. All statistical analyses were performed on R Statistical Software [29]. Statistical significance was set at $P < 0.05$, and tendencies for $0.05 < P\ 0.1$.

3. Results

No multivariate outliers were detected, as confirmed by Mahalanobis distances ($\alpha = 0.001$).

3.1. Clustering results of principal components

Principal component analysis (PCA) resulted on two major eigenvectors that explained 59.9 % (PC1) and 22.1 % (PC2) of the data variance (Fig. 1A). The contribution and correlation of all beer varietals and variables towards the eigenvectors of the principal components are found on Supplementary Tables 1–3. The PC1 appeared to describe the energy/potential fiber content of the feed with NFC having a weighed contribution and correlation values of 40.34 % and −0.98, apNDFom of 30.39 % and 00.73, EE of 17.32 % and 0.64, and CP of 11.94 % and 0.53, respectively. The PC2 appears to describe the protein content of the BSG. The CP had a weighed contribution and correlation of 74.26 % and 0.81, respectively, followed by apNDFom with a contribution of 23.77 and 0.21, respectively, where correlations and contributions of EE and NFC were insignificant. Clustering analysis identified four distinct BSG

groups that split along the multivariate biplot as energy and protein feedstuffs Fig. 1B. Clusters 1 (CL1) had higher energy and average protein content, cluster 2 (CL2) had higher NFC but lower CP, cluster 3 (CL3) had low CP and average energy content, and cluster 4 (CL4) had balanced CP and energy representing average BSG values (Fig. 1A and B).

3.2. Grain composition

The chemical composition of BSG exhibited significant variability across the 29 microbrewery varietals (Table 1), and of the clusters on Table 2. For CP, the BSG ranged from 13 to 23 % DM basis (up to 100 % variation). The energy from BSG, represented as NFC, EE, apNDFom, varied from 14 to 47 % (up to 250 % variation), 8–13 % (up to 75 % variation), and 21–45 % (up to 100 % variation), respectively. When examining clusters (CL), the apNDFom (around 100 % variation) was the highest for CL1 followed by CL3, CL4 and CL2. For ADF (10 % variation), CL4 had the highest value followed by CL1, CL3 and CL2. For CP (25 % variation), CL1 had the highest CP value, followed by CL4, CL2, and CL3. For NFC (over 100 % variation), CL2 had the highest value, followed by CL3, CL4, and CL1. For TDN all groups were within 5 % variation from 850 g/kg DM basis.

3.3. In vitro incubation

Significant time effects were observed across all fermentation parameters, except for butyrate proportion, which remained constant at approximately 10 % (Fig. 2). Gas pressure differed significantly among treatments, with the highest values recorded for CL2, followed by CL1, CL3, CL4, and alfalfa ($P < 0.001$, Table 3). For pH, alfalfa maintained the highest values, followed by CL4, CL3, CL1, and CL2, reflecting the buffering effects of the feed composition ($P < 0.001$, Table 3).

Soluble nutrient clusters (CL2, CL3, and CL4) showed the greatest reductions in pH, aligning with their higher TDN content (Fig. 3A). NH_3-N concentrations were highest for alfalfa, followed by CL2, CL1, CL4, and CL3 ($P = 0.011$, Fig. 3B). CL1 and CL2 had significantly different values for all measured parameters compared to CL4 ($P < 0.011$, Table 3). For NDF digestibility, CL1 and CL2 showed higher values than CL3 and CL4 ($P < 0.001$, Table 3), while apNDFom digestibility was also significantly different across groups, with CL1 having the highest and CL4 the lowest values ($P < 0.001$, Table 3).

3.4. Volatile fatty acids and gas composition

Total VFA production was highest in CL2, followed by CL1, CL3, and CL4, with alfalfa producing the least ($P < 0.001$, Table 3). Acetate, propionate, and butyrate proportions differed significantly among feed clusters ($P < 0.001$, Table 3), with CL2 and CL1 producing the most VFA. Butyric acid was significantly higher in all clusters compared to alfalfa ($P = 0.002$, Table 3).

Gas production dynamics revealed significant time effects for CH_4 and CO_2 production (Fig. 4A and B, respectively). Alfalfa produced the least CH_4 and CO_2, with CL4 showing significantly lower CH_4 emissions compared to the other clusters ($P < 0.001$, Table 4).

3.5. Gas production modeling

Gas production patterns were best described by a two-pool Gompertz model (Table 5, Fig. 2). The second nutrient pool (V2)
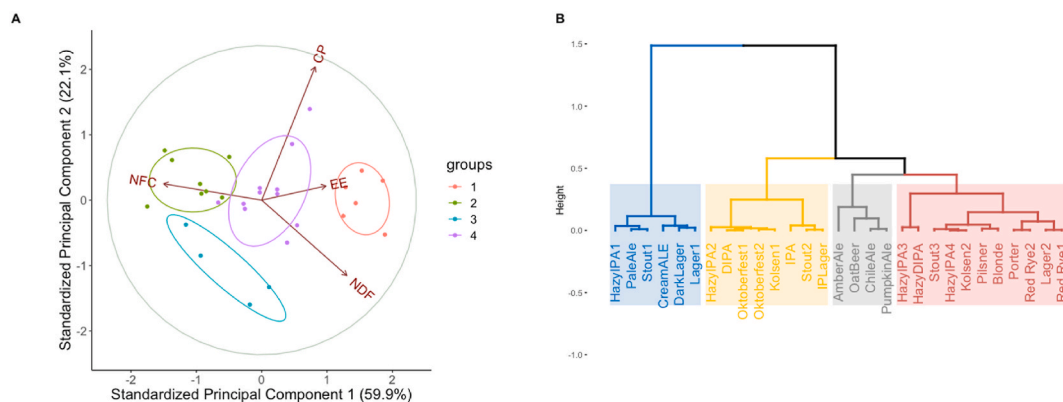

Fig. 1. (A) Hierarchical cluster classification of the principal components of the nutrient composition of 29 microbreweries spent grain varietals displaying the potential classification as protein and energy supplements. Dim1 (59.9 %) = first principal component eigenvector explaining 59.9 % of the variance representing the feed energy and fiber variation of the grains, Dim2 (22.1 %) = second principal component eigenvector explaining 22.1 % of the variance of the nutrient composition of the spent microbreweries' grains representing the feed protein variation. Cluster 1 = Oktoberfest, Double Indian Pale Ale (IPA), Pale Ale, Indian Pale Lager, Oktoberfest, IPA, Stout, Kolsen; Cluster 2 = Pepper beer, Oat beer, Pumpkin Ale, Amber Ale; Cluster 3 = Blonde, Porter, Red Rye, Stout, Hazy IPA, Pilsner, Kolsen, Red Rye, Hazy IPA, Double IPA, Lager; Cluster 4 = Pale Ale, Hazy Pale Ale, Mexican Lager, Cream Ale, Stout, Dark Lager. (B) Agglomerative hierarchical clustering of the beers split into 4 clusters.

Table 2

Proximate chemical composition reported in dry matter basis for the microbreweries spent grains.

Parameter[a] g/kg DM basis	Analytical fraction[b], g/kg DM basis							
	DM	OM	apNDFom	ADF	EE	CP	NFC	TDN
Alfalfa	916.0	911.0	431.0	377.0	16.7	198.0	350.1	624.0
CL1	953.8	965.6	443.9	161.6	115.6	219.7	186.5	838.4
CL2	958.8	967.2	274.9	142.2	92.8	192.9	406.5	881.2
CL3	955.8	965.5	363.3	146.5	102.6	144.7	354.9	866.5
CL4	952.6	961.7	343.9	166.4	107.4	200.4	310.0	865.0

[a] CL = cluster generated from hierarchical cluster from principal components of the nutrient profile of the individual microbrewer's spent grains, CL1 = cluster group 1, CL2 = cluster group 2, CL3 = cluster group 3, CL4 = cluster group 4.

[b] DM = dry matter, OM = organic matter, apNDFom = neutral detergent fiber exclusive of residual ash and protein, ADF = acid detergent fiber exclusive of residual ash, EE = ether extract, CP = crude protein, NFC = non-fibrous carbohydrates, TDN = total digestible nutrients.
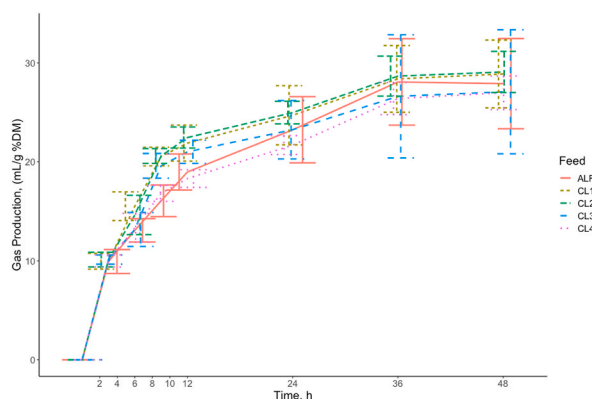

Fig. 2. Gas production in function of the time and the feed clusters and alfalfa for the experiment. Solid grey line is Alfalfa, green dashed line is the cluster 1 (CL1), the blue line with two dashes (—) represents the second cluster (CL2), the black dotted line represents the third cluster (CL3), and the red dot followed by a dash is the fourth cluster (CL4).

Table 3

In vitro digestibility parameters, and volatile fatty acid production in function of microbreweries spent grain varietals.

Item[a]	Parameter Estimates[b]					SE[c]	P-values[d]		
	CL1	CL2	CL3	CL4	ALF		Cluster	Time	C × T
Pressure psi/g DM	3.24[a]	3.29[a]	3.05[ab]	2.88[b]	2.82[b]	0.397	<0.001	<0.001	0.206
pH	6.91[a]	6.90[a]	7.00[b]	7.12[c]	7.21[c]	0.082	<0.001	<0.001	<0.001
NH₃-N, mg/dL	18.2[a]	18.50[a]	16.80[b]	17.80[a]	20.20[c]	0.912	0.011	<0.001	0.328
NDFd %	63.2[a]	59.80[b]	58.20[b]	54.40[c]	58.40[b]	1.882	<0.001	<0.001	0.299
mmol/g DM									
Total VFA	63.1[a]	66.5[ac]	60.3[ab]	58.2[b]	56.8[b]	4.55	<0.001	<0.001	0.233
Acetic	38.3[ab]	40.0[a]	38.3[ab]	36.5[b]	38.7[a]	2.39	<0.001	<0.001	0.037
Propionic	10.86[ab]	11.79[a]	10.69[ab]	9.67[b]	8.60[b]	1.64	<0.001	<0.001	0.269
Isobutyric	3.16[a]	3.70[b]	3.75[b]	3.33[a]	3.14[a]	0.39	<0.001	0.002	0.030
Butyric	8.26[ab]	9.34[a]	7.53[b]	6.32[c]	4.30[d]	0.99	<0.001	<0.001	0.501
Isovaleric	1.41[a]	1.47[a]	1.58[a]	1.57[a]	1.74[b]	0.18	<0.001	<0.001	0.542
Valeric	1.42[a]	1.44[a]	1.50[a]	1.46[a]	1.19[b]	0.12	<0.001	<0.001	<0.001
A:P ratio	3.76[a]	3.50[b]	3.68[ab]	3.88[a]	4.66[c]	0.19	<0.001	0.075	0.318
Proportions, g/kg DM									
Acetate	607.0[a]	594.0[b]	610.0[a]	625.0[c]	679.0[d]	10.50	<0.001	0.021	0.498
Propionate	170.0[a]	172.0[a]	166.0[a]	162.0[a]	146.0[b]	9.62	<0.001	<0.001	0.796
Butyrate	126.9[a]	133.0[b]	118.4[c]	106.4[d]	72.9[e]	5.22	<0.001	0.540	0.013

[a] VFA = volatile fatty acids, AP = acetate to propionate ratio, NDFd = Neutral detergent fiber digestibility, NH₃-N = ammonia nitrogen.

[b] CL = cluster generated from hierarchical cluster from principal components of the nutrient profile of the individual microbrewer's spent grains, CL = cluster generated from hierarchical cluster from principal components of the nutrient profile of the individual microbrewer's spent grains, CL1 = cluster group 1, CL2 = cluster group 2, CL3 = cluster group 3, CL4 = cluster group 4, ALF = alfalfa.

[c] SE = standard error of the mean.

[d] Statistical significances declared at 0.05, trends evaluated at 0.1. Letter superscripts represent the estimated marginal mean differences across feed clusters, different letters across clusters indicate statistical difference.
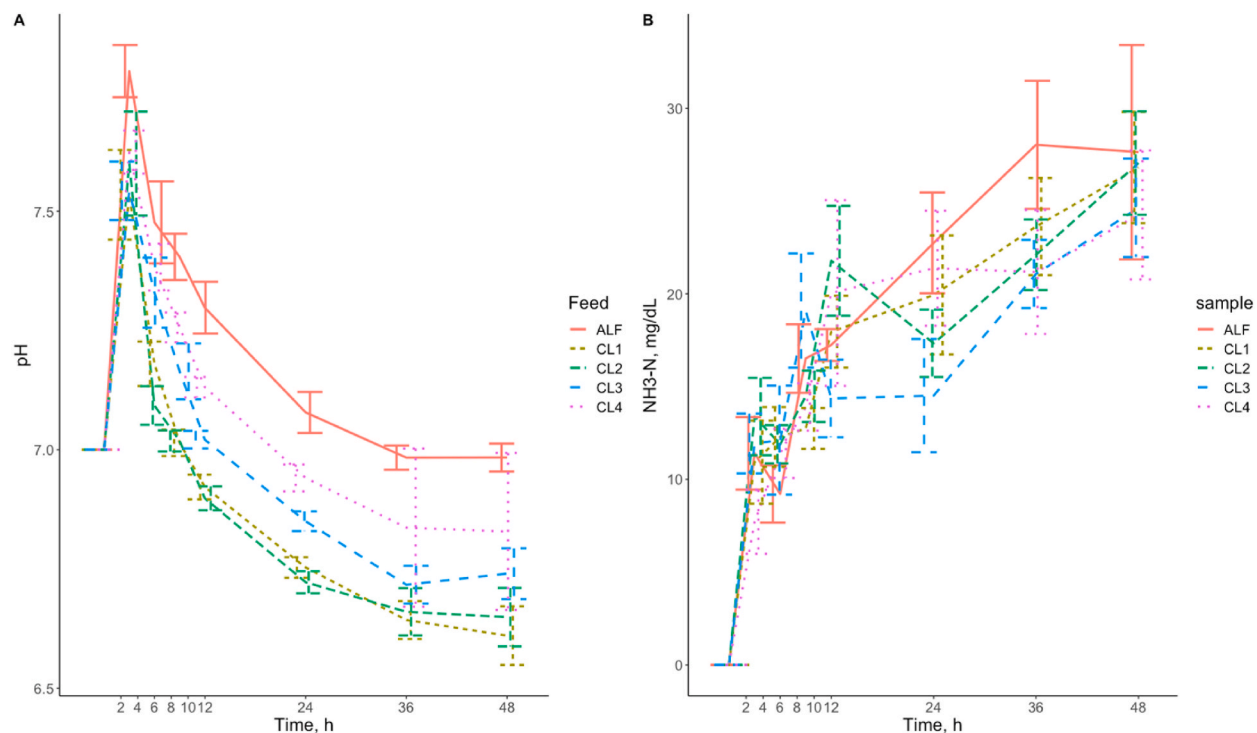
Fig. 3. (A) pH and (B) NH₃-N as a function of the time and the feed clusters and alfalfa incubated for the experiment. Solid grey line is Alfalfa, green dashed line is the cluster 1 (CL1), the blue line with two dashes (– -) represents the second cluster (CL2), the black dotted line represents the third cluster (CL3), and the red dot followed by a dash is the fourth cluster (CL4).
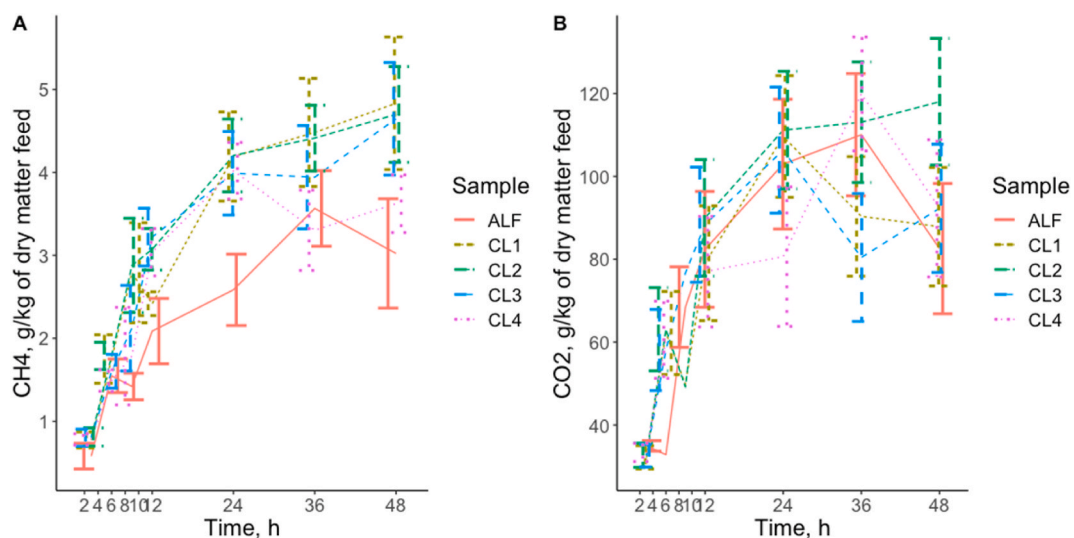

Fig. 4. (A) Methane gas production as a function of the time and the feed clusters and alfalfa for the experiment. Solid grey line is Alfalfa, green dashed line is the cluster 1 (CL1), the blue line with two dashes (—) represents the second cluster (CL2), the black dotted line represents the third cluster (CL3), and the red dot followed by a dash is the fourth cluster (CL4). (B) Carbon dioxide gas production in function of the time and the feed clusters and alfalfa for the experiment. Solid grey line is Alfalfa, green dashed line is the cluster 1 (CL1), the blue line with two dashes (– -) represents the second cluster (CL2), the black dotted line represents the third cluster (CL3), and the red dot followed by a dash is the fourth cluster (CL4).

Table 4

In vitro enteric methane (CH_4) and carbon dioxide (CO_2) production as a function of microbreweries spent grains.

Item	Parameter Estimates[a]					SE[b]	P-values[c]		
	CL1	CL2	CL3	CL4	ALF		Cluster	Time	C × T
g/kg of feed									
CO_2	80.8	78.9	81.3	83.6	85.5	21.7	0.910	<0.001	0.989
CH_4	2.79[ab]	2.94[ab]	2.83[ab]	2.44[ab]	2.19[a]	0.259	<0.001	<0.001	0.689
g/kg of digestible feed									
CO_2	130	133	142	156	148	36.5	0.286	<0.001	0.980
CH_4	4.42[ab]	4.81[ab]	4.85[ab]	4.48[ab]	3.75[a]	0.437	0.007	<0.001	0.865

[a] Parm = parameters, CL = cluster generated from hierarchical cluster from principal components of the nutrient profile of the individual microbrewer's spent grains, CL1 = cluster group 1, CL2 = cluster group 2, CL3 = cluster group 3, CL4 = cluster group 4, ALF = alfalfa.

[b] SE = standard error of the mean.

[c] Statistical significances declared at 0.05, trends evaluated at 0.1. Time comparisons were evaluated for 0, 3, 6, 9, 12, 24, 36, 48 h. Letter superscripts represent the estimated marginal mean differences across feed clusters, different letters across clusters indicate statistical difference.

exhibited significant differences in volume ($P < 0.001$, Table 5) and degradation rate (C2, $P = 0.007$, Table 5), with CL1 showing the highest degradation rates and CL4 the lowest.

4. Discussion

The current study investigates overlooked variation in composition of BSG and the results showed that BSG differed nutritionally across breweries and beer-styles and showed distinct fermentation patterns and dynamics. Limitations in the present study include the inability to complete a full feeding trial because of grain availability, nonetheless, the *in vitro* fermentation and chemical composition offer great initial insights into future works and valuable information regarding how to classify and use BSG as livestock feed. Ruminal microbes depend on the interaction between energy and protein; too much energy and low CP can decrease the digestibility of carbohydrates in the diet, whereas low energy and high CP can increase the losses of protein [30]. Such interactions highlight the importance of not overlooking variation in chemical composition of BSG and other novel by-products which may be incorporated into livestock diets.

High variations between and within the BSG of different breweries was detected, with more significant variation for NFC (250 %), with much higher values for Ales ($\cong$ 400 g NFC/kg DM) compared to Lagers ($\cong$ 250 g NFC/kg DM) and with the highest NFC presented by the IPAs ($\cong$ 480 g NFC/kg DM) and Stouts ($\cong$ 460 g NFC/kg DM). Variation in NFC is of critical importance for ruminant diets since NFC are rapidly fermented in the rumen, and this can lead to rapid production of VFA and consequently increase the energy available to the animal [31]. Non-fibrous carbohydrates composed of starch, sugars, and pectin are readily available energy sources aiding to cattle's weight gain [32]. Usually, care in the formulation of this nutritional entity provides suitable substrates that promote faster production cycles and weight gains. High levels of NFC are generally related to higher protein in both meat and milk. This is because the presence of sources of rapidly fermented energy used in the rumen enhances the growth of microorganisms that produce a lower acetic:propionic acid ratio [33].

The variation in apNDFom content can be significant when choosing the roughage to be mixed into the diet. BSG with a higher percentage of apNDFom (as Mexican Lager with $\cong$ 500 g/kg DM), is more slowly degraded by ruminal microorganisms, producing the fatty acids acetate and butyrate [34]. These VFAs play an essential role in maintaining rumen health, providing energy to the animal, and regulating rumen pH. Compared to alfalfa, BSG presented less fiber and more NFC, which provided an enhanced VFA with energetic potential (propionate and butyrate) and, consequently, total VFA. Which indicate that BSG alone may not always be a sufficient fiber source.

Mischaracterizing BSG as protein sources alone carries significant consequences nutritionally. Considering the grains just as protein supplementation is misleading since variations in energy and protein were witnessed in our clusters. In fact, even within same beer styles, three stouts (beers 6, 14, and 28), and two double India pale ales (beers 5 and 20) were clustered separately. A potential explanation is that beer number 20 is a hazy India pale ale which utilize higher protein to allow for specific flavour profiles attributed to the polyphenolic bonds they form [35]. Likewise, the stout number 28 which was an experimental 100 % oatmeal beer which explained its separate clustering. The clustering of the stout number 6, an American imperial stout, and stout number 14, a sweet milk stout is explained because of longer fermentation time and higher alcohol content on stout number 6. These variations suggest that BSG can serve both as energy and protein sources, depending on their chemical composition.

Cluster analysis demonstrated a strong association of energy-related factors (apNDFom, EE, NFC) with clusters CL1 and CL4, indicating potential applications as energy-rich feeds. For instance, CL4 combined high energy and protein, while CL1 exhibited high protein with lower energy. These findings emphasize the importance of a nuanced classification of BSG for diet formulation to optimize livestock productivity and minimize nutrient imbalances [30].

The observed differences in gas production, digestibility, and VFA profiles align with the compositional variability across clusters. Fermentation patterns followed a two-pool Gompertz model, with distinct phases reflecting rapid fermentation of soluble substrates (4–12 h) and slower fermentation of fiber fractions (36 h) [36,37]. The delayed fermentation in alfalfa and CL2 may be attributed to pre-brewing processing or complex nutrient interactions, such as Maillard reactions, reducing microbial substrate accessibility [38].

In vitro fermentation results underscore the relationship between nutrient solubility and pH dynamics. CL1, with more soluble

Table 5

In vitro gas production nonlinear estimates for equation parameters in function of the time and the microbreweries spent grains.

Item[a]	Parameter Estimates[b]					SE[c]	*P*-value[d]
	CL1	CL2	CL3	CL4	ALF		
V1	5.07	4.99	6.68	8.07	3.40	4.681	0.8181
V2	23.74[a]	22.60[ab]	21.68[ab]	17.76[b]	24.13[a]	6.128	<0.001
C1	0.01	0.01	0.01	0.01	0.01	0.018	0.492
C2	0.10[ac]	0.13[ab]	0.11[a]	0.14[b]	0.07[c]	0.038	0.007
L	0.21	0.50	0.25	0.34	0.01	0.325	0.915

[a] V1, C1 and L = the asymptotic cumulative gas volume, rate and lag parameters for the first pool; V2 and k2 = the asymptotic cumulative gas volume and rate parameters for the second pool.

[b] CL = cluster generated from hierarchical cluster from principal components of the nutrient profile of the individual microbrewer's spent grains, CL1 = cluster group 1, CL2 = cluster group 2, CL3 = cluster group 3, CL4 = cluster group 4, ALF = alfalfa.

[c] SE = standard error of the mean.

[d] Statistical significances declared at 0.05, trends evaluated at 0.1. "-".

nutrients, led to faster pH declines, while the higher fiber content in CL2 slowed the pH drop. NH_3-N concentrations are essential in understanding fermentation dynamics and their efficiency. NH_3-N varied significantly, with CL3 showing the lowest values, indicative of lower protein content and reduced nitrogen excretion potential. Efficient nitrogen utilization remains critical to reducing environmental nitrogen losses from livestock systems [39] which is also important considering that higher nitrogen excretion is also associated with greater water use by animals [40]. Fiber degradation, represented by apNDFom digestibility, was highest in CL1 but significantly lower in CL4, likely due to antinutritional properties of BSG such as their lignification [41]. These results highlight the need to balance BSG with other diet components to maintain rumen pH and avoid acidosis, particularly for low-fiber BSG.

The observed differences in VFA production further support the dietary potential of BSG. CL2 produced the highest total VFA, with butyrate levels significantly higher in all BSG clusters compared to alfalfa. This suggests a potential role for BSG in enhancing rumen development in young ruminants, as butyrate is a critical driver of rumen growth and functionality [42–44]. These findings reinforce the importance of considering both protein and energy contributions when integrating BSG into livestock diets.

Lastly, the environmental implications of feeding BSG are also notable. Methane production was lower in CL4 suggesting potential for reduced greenhouse gas emissions when incorporating BSG into diets. As CH_4 serves as a hydrogen sink during VFA production in the rumen [45], the differences we observed highlight the need for tailored feeding strategies to optimize hydrogen utilization and minimize environmental impacts.

Overall, the variability in BSG composition underscores the need for careful classification to maximize their nutritional potential while minimizing environmental footprints. These findings provide a foundation for sustainable use of BSG in livestock diets, supporting both productivity and environmental goals. Future research should focus on experimental validation in livestock, particularly assessing the impact of BSG on amino acid and fatty acid profiles and their effects on the flavour and nutritional quality of meat and milk. Additionally, economic analyses should evaluate transportation costs and optimal brewery-to-farm distances to justify the inclusion of BSG in diets. Ensuring accurate classification and utilization of BSG and other by-products is essential to avoid over-generalization and maximize their potential as sustainable feed resources.

5. Conclusions

This study highlights the significant variability in the chemical composition and fermentation dynamics of BSG from craft microbreweries, challenging the traditional classification of BSG as a protein feed alone. Multivariate analyses revealed distinct nutrient-based clusters, with CP ranging from 13 to 23 % DM and NFC from 14 to 47 %, corresponding to up to 250 % variation in energy content. These clusters demonstrated high-energy, low-energy, and moderate-protein groups, providing valuable insights for precision diet formulation. In vitro fermentation patterns showed that nutrient variability influenced gas production, pH, and VFA profiles. BSG with higher soluble nutrient content (e.g., NFC) resulted in faster fermentation rates and pH declines, while more fibrous BSG (apNDFom up to 45 %) exhibited slower fermentation. Methane production varied significantly, with lower emissions observed in high-fiber clusters, supporting the potential for BSG to reduce environmental impacts when used strategically. These findings underscore the importance of careful BSG classification for livestock diets. Incorporating BSG into precision feeding strategies could enhance the nutrient density of protein, energy, or both, while supporting the development of specialty animal products in beef and dairy operations. Variability even among similar beer styles (e.g., stouts, IPAs) suggests the need for targeted inclusion to optimize production efficiency and product quality.

CRediT authorship contribution statement

Arturo Macias Franco: Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Aghata E.M. Silva:** Writing – review & editing, Investigation. **Tio Brody:** Investigation. **Graham Holton:** Writing – review & editing. **Macy Rockwell:** Investigation. **Nelcino F. De Paula:** Writing – review & editing, Visualization. **Leilson R. Bezerra:** Writing – review & editing, Resources, Investigation, Data curation. **Mozart A. Fonseca:** Writing – review & editing, Validation, Supervision, Project administration,

Methodology, Funding acquisition, Formal analysis, Conceptualization.

Data availability

The data sets that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval

The animals used in this experiment were cared for according to guidelines approved by the Institutional Animal Care and Use Committee University of Nevada, Reno (protocol #00738).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e41606.

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