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In vitro evaluation of *Saccharomyces cerevisiae* cell wall fermentability using a dog model

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Summary

Six *Saccharomyces cerevisiae* cell wall samples were tested by the in vitro gas production technique using dog faeces as *inoculum*. In particular, the substrates resulted from three different production processes (alcoholic_A, bakers_BA and brewers_BR) and were characterized by two different carbohydrates (mannans + glucans) concentrations nitrogen-free extract (NFE high and low). Gas production of fermenting cultures was recorded for 72 hr to estimate the fermentation profiles. The organic matter degradability (OMD), fermentation liquor pH, short-chain fatty acids (SCFA) and ammonia (NH_3) productions were also measured. All substrates presented a high percentage of OMD (>92%) and moderate fermentability in terms of cumulative volume of gas related to incubated OM (OMCV >50 ml/g) and short-chain fatty acids production (>25 mmol/g), proving their functional properties. Comparing the substrates, it seems evident that the production process affects the chemical composition of the yeast cell wall in terms of crude protein, ether extract and ash content. Consequently, the in vitro fermentation process was significantly different among substrates for volume of gas, SCFA and ammonia production. Regarding the fermentation rate profiles, the production process influenced mainly the curve shape, whereas the NFE concentration affected the quantity of gas produced per hour. In particular, both *S. cerevisiae*_BR showed very high percentage of OM degradability, gas and SCFA productions and a fast fermentation process due to their high content of fermentable carbohydrates. On the contrary, both *S. cerevisiae*_BA yeast cell walls appeared to be less degradable and fermentable, probably due to their high content of ether extract. Regarding both *S. cerevisiae*_A, the high protein content of these substrates could explain the contrasting in vitro results (high degradability with low gas and SCFA production).

KEYWORDS
ammonia, gas production, pre-biotic, short-chain fatty acids, yeast

1 | INTRODUCTION

The yeast cell walls are considered important sources of protein, vitamins and polysaccharides such as glucose, mannose and N-acetyl-glucosamine. The *Saccharomyces cerevisiae* (SC) is a unicellular eukaryotic, which converts carbohydrates to carbon dioxide and alcohols by fermentation. Thus, it has been used in baking and in alcoholic beverages production for thousands of years (Legras, Merdinoglu, Cornuet, & Karst, 2007).

Yeasts are principally obtained from the production of bakers and brewers, or after alcoholic production. Yeast extracts are obtained mechanically after autolysis by acid or alkali hydrolysis or by enzymatic treatment

(Borchani et al., 2014; Freimund, Sauter, Käppeli, & Dutler, 2003). The extraction yield for SC varies for amount of nutrients (i.e., protein, carbohydrates and lipid contents) (Freimund et al., 2003), and purity in function of the quality of the utilized yeast cell walls (Liu, Wang, Cuic, & Liu, 2008).

The yeast cell wall contains β-glucans, mannan oligosaccharides (MOS), proteins, lipids, chitin and vitamins (Jaehrig et al., 2008) in different proportion. In particular, β-glucans are the main polysaccharides of yeast, representing about the 24% of dry matter, and are characterized by a complex structure. Several β-glucans have insoluble branched β-(1-3) bond, and they are structural components of cell wall, whereas the minor constituents have soluble branched β-(1-6) bond and are essential for the cross linking (Borchani et al., 2016; Magnani et al., 2009). The β-glucans have the ability to remain intact in the digestive tract, and, because of their specific properties (i.e., oil binding, water holding capacity and emulsion stability), are frequently utilized in food/feed industry (Barriga, Cooper, Idziak, & Cameron, 1999; Cameron, Cooper, & Neufeld, 1988).

The mannan oligosaccharides constitute about 14% of the dry weight of yeast (White, Newman, Cromwell, & Lindemann, 2002). Most of the proteins are linked to the mannans, and they are referred as mannan-protein complex, which, in small quantities, bounds glucose, galactose and xylose (Lipke & Ovalle, 1998).

Both β-glucans and MOS could be considered as pre-biotic in human as well as in animals (Chen & Seviour, 2007; Hai & Fotedar, 2009; Laroche & Michaud, 2007), supporting the immune system (Newman & Newman, 2001; O'Quinn, Funderburke, & Tibbetts, 2001) and reducing the intestinal pathogen colonization (Johnson et al., 2012). Furthermore, by favouring the growth and development of intestinal microorganism, β-glucans protect the body from infections (i.e., viral, fungal or bacterial) and immunosuppression caused by stress (Borchani et al., 2014; Liu, Wang, & He, 2011; Magnani et al., 2009).

The US Food and Drug Administration recognize cells of SC and/or cell extracts as safe products to use as supplements in animal diets (Rodriguez, Kildegaard, Li, Borodina, & Nielsen, 2015). Because of their well-known benefits, many different processes have been developed to obtain a better quality of the extract by maintaining their native structure.

The aim of this study was to compare six yeast cell wall samples obtained by *S. cerevisiae* differing in production process and carbohydrates concentration. At this aim, the in vitro gas production technique (IVGPT) using dog faeces as *inoculum* was employed to study fermentation characteristics and kinetics. The hypothesis was that the production methods or carbohydrates concentration can affect the chemical characteristics of the *S. cerevisiae* cell wall samples as well as their fermentability in dog hindgut. Consequently, it is possible to hypothesize different applications in dog nutrition.

2 | MATERIALS AND METHODS

Six samples of *S. cerevisiae* cell walls obtained by three different production processes: alcoholic-A, yeast grown in a molasses basis through an anaerobic process; brewers-BR, yeast grown in a cereal basis through an anaerobic process; bakers-BA, yeast grown in a molasses basis through an aerobic process (no limitation of oxygen). Subsequently, each sample was hydrolysed to break cell wall and centrifuged in order to concentrate carbohydrates. A single sample of each "production process," chosen raising information about different batches produced during an year and was representative of a mean of low and high concentration of carbohydrates, in function of the production process.

The concentration of yeast carbohydrates (mannans and glucans) was determined at the laboratories of Biorigin (São Paulo, Brazil) according to the protocol proposed by Freimund, Janett, and Arrigoni (2005).

At the Feed evaluation laboratory of the Department of Veterinary Medicine and Animal Production (Napoli, Italy), the proximate analysis of the samples was performed according to AOAC (2005) procedures: crude protein ID 954.01; ether extract ID 920.39C; ash ID 942.05; dry matter ID 934.01. To study the fermentation characteristics of the yeast cell walls, a trial was performed using the in vitro gas production technique (IVGPT) with the faeces from dogs as *inoculum*. Each SC sample was accurately weighed (mean ± *SD*: 0.5032 ± 0.0019 g) in quadruplicate into 120-ml serum flasks sealed with butyl rubber stoppers and aluminium crimp. To guarantee the adequate environmental condition for the microbial population, a medium composed by nitrogen, fatty acids, vitamins and minerals was added under anaerobic condition to each flask. Aneaerobiosis was guaranteed by the continuous CO_2 insufflation and by the presence of a reducing agent (1 ml of a solution of L-cysteine hydrochloride, NaOH 1 M and sodium sulphide) (Calabrò et al., 2013).

Faeces were collected from six healthy adult crossbreed dogs (mean age: 4.5 ± 1.3 years; mean live weight 25 ± 6 kg) progressively adapted (10 days) to a commercial standard diet (crude protein 22% a.f., crude fibre 5.0% a.f.) and daily administered according to the equation 130 kcal/$kg^{0.75}$ (NRC, 2006). After collection, faecal samples were immediately transported, in thermostated boxes under anaerobic condition, to the laboratory, and they were processed within 40 min. Faecal samples were pooled, diluted (1:10 v:v) with 0.9% NaCl sterile solution, homogenized and filtered through six layers of gauze and added to each flask (5 ml), that were placed in an incubator (PI400 Carbolite, Sheffield, UK) at 39°C for 72 hr (Cutrignelli, 2007). Gas production of fermenting cultures was recorded with a manual pressure transducer (Cole and Parmer Instrument, Vernon Hills, IL, USA) after 3, 6, 9, 12, 15, 18, 21, 24, 28, 32, 37, 44, 48, 56, 69 and 72 hr of incubation. The cumulative volume of gas obtained for each sample at 72 hr was related to the quantity of incubated organic matter (OMCV, ml/g). The fermentation was stopped by cooling the flasks at 4°C, and the pH of fermenting liquor was measured (pH meter model 3030; Alessandrini Instrument SpA glass electrode Jenway, Dunmow, UK). Two aliquots (5 ml) of fermenting liquor were collected and frozen at −15°C for the end products analysis: short-chain fatty acids (SCFA) and ammonia (NH_3). In particular, SCFA (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) were determined by gas chromatography (ThermoQuest Italia SpA, Rodano, Milan, Italy; model. Focus) as indicated by Musco et al. (2016). To evaluate the degree of proteolysis that occurs during the fermentation, the branched chain

fatty acids were calculated according to the following equation: (BCFA = isobutyrate + isovalerate/SCFA). Ammonia production was determined colorimetrically (Thermo Scientific, Helios Gamma 110-240 Volts, Waltham, MA, USA) at wavelength of 623 nm according to Searle (1984). Flask residues were filtered through pre-weighed sintered glass crucibles (porosity #2, DURAN Group GmbH Mainz, Germany), and residual organic matter was determined by burning the sample at 550°C for 5 hr. Organic matter degradability (OMD, %) was calculated as difference between incubated and residual OM. Four flasks were incubated without substrate (blank) to correct OMD, gas production and end products values.

The gas profile of each flask was fitted to the sigmoid model described by Groot, Cone, Williams, Debersaques, and Lantinga (1996) as follows: $G = A/(1 + B/t)^C$, where G is the total gas produced (ml/g of incubated OM) at t (hr) time; A is the asymptotic gas production (ml/g of incubated OM); B is the time at which one-half of the asymptote is reached (hr); C is the switching characteristic of the curve. The model parameters were utilized (Bauer, Williams, Voigt, Mosenthin, & Verstegen, 2001) to calculate the fermentation rate profile according to the following formula: $(A \cdot C^B) \cdot B \cdot [\text{time}^{(-B-1)}]/[(1 + C^B) \cdot (\text{time}^{-B})^2]$.

All the fermentation characteristics obtained (OMCV, OMD, SCFA, BCFA, pH and NH_3) were subjected to analysis of variance using the JMP software (SAS Institute, NC, USA, 2014) to detect the differences among the production process and NFE concentration according to the following equation:

$$y_{ij} = \mu + PP_i + NFE_j + (PP * NFE)_{ij} + \varepsilon_{ij}$$

where y, is the experimental data; μ, the general mean; PP, the production process (i = alcoholic, brewers, bakers); NFE concentration (j = high, low); ε, the error term.

When significant differences among substrates were found by the analysis of variance, means were compared using the t test using the JMP software (SAS Institute, NC, USA, 2014).

3 | RESULTS

3.1 | Chemical composition

In Table 1, the chemical composition of the tested yeast cell walls is reported. The production process affected ether extract, ash and NFE levels. The lipid amount was more than three times higher in baker's SC cell wall than in the other substrates. *Saccharomyces cerevisiae* grown on brewers showed the highest dry matter and ash contents. The different NFE concentration between yeast cell walls obtained after alcoholic production process is due to the higher crude protein content of SC-A that is low in NFE (33.7 vs. 24.3% a.f.), while it is due to the slight variations in ether extract (3.58 vs. 2.86% a.f.) for SC-BR and in crude fibre (2.03 vs. 1.18% a.f.) for SC-BA.

The sum of glucans + mannans (data not showed) represents more than 49% of the composition for all the tested samples, even if it was affected by the yeast cell wall production process (SC-BA high and low NFE: 65.6% and 55.7% a.f.; SC-BR high and low NFE: 60.9% and 60.0% a.f.; SC-BA high and low NFE: 63.2% and 49.1% a.f.).

3.2 | In vitro fermentation characteristics

The main parameters of in vitro fermentation characteristics after 72 hr of incubation in function of production process and NFE concentration are reported in Table 2. The production process and the NFE concentration significantly ($p < .001$) affected OMD and OMCV, whereas the interaction was significant only for OMCV. All the tested samples showed a OMD always higher than 92%, varying from 92.7% of SC-BA-low NFE to 97.3% of SC-A-high NFE. The cumulative gas production was significantly affected by production process, even if with a different trend compared to OMD. In particular, the SC-A-high NFE showed the highest OMD value and the lowest OMCV one. The NFE concentration directly influenced ($p < .01$) the OM degradability and gas production.

The fermentation rate profiles of the tested substrates, depicted in Figure 1, in the first 24 hr of incubation put in evidence the differences due to both effects. It seems evident that the production process influenced the curve shape, whereas the NFE concentration affected the quantity of gas produced per hour. In particular, both SC-BR yeast cell walls showed very fast fermentation kinetics, while SC-BA fermented slowly.

As reported in Table 3, most of in vitro fermentation end products (acetate, propionate, valerate, SCFA, BCFA, ammonia and pH) were affected by the extract production process, but only the isobutyrate ($p < .05$) and valerate ($p < .01$) resulted influenced by NFE concentration. The acetate, propionate and total SCFA values were the highest

TABLE 1 Chemical composition of the tested substrates

Sample	*n*	DM	CP	EE	CF	Ash	NFE
SC-A-high NFE	4	94.1	24.3	3.86	1.12	3.0	61.82
SC-A-low NFE	4	93.0	33.7	1.99	1.08	1.0	55.23
SC-BA-high NFE	4	93.4	23.8	11.37	1.18	2.0	55.05
SC-BA-low NFE	4	92.8	23.9	11.84	2.03	2.9	52.13
SC-BR-high NFE	4	96.1	22.1	2.86	1.33	5.3	64.54
SC-BR-low NFE	4	96.0	21.7	3.58	1.27	7.3	62.54

SC-A, *Saccharomyces cerevisiae* alcoholic extract; SC-BR, *Saccharomyces cerevisiae* brewer's extract; SC-BA, *Saccharomyces cerevisiae* bakers extract; DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fibre; NFE, nitrogen-free extract.

TABLE 2 In vitro fermentation characteristics of *Saccharomyces cerevisiae* in function of production process and NFE concentration

Sample	*n*	OMD (%)	OMCV (ml/g)
Production process effect			
SC-A	8	96.48^{A}	51.22^{C}
SC-BR	8	95.81^{AB}	112.89^{A}
SC-BA	8	94.30^{B}	64.86^{B}
NFE concentration effect			
High	12	96.55^{A}	79.46^{A}
Low	12	94.52^{B}	73.19^{B}
Interaction		0.2255	0.0024
RMSE		1.20	4.83

SC-A, *Saccharomyces cerevisiae* alcoholic extract; SC-BR, *Saccharomyces cerevisiae* brewer's extract; SC-BA, *Saccharomyces cerevisiae* bakers extract; OMD, organic matter degradability (% of incubated OM); OMCV, cumulative volume of gas related to incubated OM (ml/g); RMSE, root mean square error.
Along the column for each effect: A, B = $p < .01$.

in the brewers SC cell wall (22.05, 7.09 and 33.81 mmol/g, respectively $p < .01$), whereas, NH_3 production resulted the highest in SC-BA (71.60 mg/L; $p < .01$). *Saccharomyces cerevisiae* alcoholic cell wall showed the highest pH value (6.66; $p < .01$).

4 | DISCUSSION

Yeast cell wall has been considered a valuable pre-biotic in dog nutrition, in particular, cell wall of SC, being a source of β-glucans and MOS, is able to inhibit pathogenic bacteria through several different mechanisms and to favour gut health (Swanson & Fahey, 2006). Elghandour et al. (2014) reported that SC provides important nutrients or nutritional co-factors such as vitamins (e.g., biotin and thiamine) and stimulates microbial growth and activity. Indeed, commercial products are very different for both production methods and for material from which they come from (Freimund et al., 2005).

Regarding chemical composition, it has been reported (Pinto, Coelho, Nunes, Brandão, & Coimbra, 2015) that the raw materials and the methods used for the extract preparation of yield affect some nutritional parameters (i.e., protein content and carbohydrates fermentability) as well as the amount of yeast cell wall. Consequently, it is difficult to compare the results of this investigation with other reported in literature. For example, in a previous study, Calabrò et al. (2013) used a commercial *S. cerevisiae* cell wall product, that appeared similar to the tested SC alcoholic extract for protein level (28.8% a.f.), but showed lower lipids amount (0.80% a.f.). Commercial supplement based on inactive dried yeast is usually richer in protein content than the yeast cell wall product because of the nutrients present into the cytoplasm. Rodriguez et al. (2015) described a different chemical composition for an inactive brewer's yeast particularly rich in crude protein and selenium (47.0% and 0.3% DM, respectively) and a commercial yeast cell wall product characterized by the following composition: crude protein 14% DM, ether extract 20% DM, β-glucans 24% DM, mannose 22% DM. In this study, the lipid content of SC-BA (more than 11% a.f.) and the ash content of SC-BR (6.3% a.f.) were particularly high.

There are only few data in the literature regarding the in vitro fermentation characteristics and profile of SC cell wall obtained by gut microbiota of dog, and they are not always comparable and consistent. Respect to the present study, Calabrò et al. (2013) found higher values for OMD (99.1%) and OMCV (199 ml/g) and lower SCFA values (17.89 mmol/g) incubating for 48 hr purified *S. cerevisiae* cell wall with dog faecal *inoculum*. Probably, the different incubation length (48 vs. 72 hr), as well as the different chemical characteristics of the tested substrates, influenced these results. The choice of a longer incubation time in this study was due to the specific substrates studied (prebiotics). The aim was to compare the fermentation kinetics of different yeast cell walls, and consequently, the asymptote of in vitro gas production had to be reached, after 48 hr of incubation some substrates had reached the maximum rate of fermentation (Figure 1).

The pH, being for all the tested substrates in the range indicated by Younes et al. (2001) as the physiological pH of colonic contents and resulting faeces in carnivores, confirms that in the flasks there were

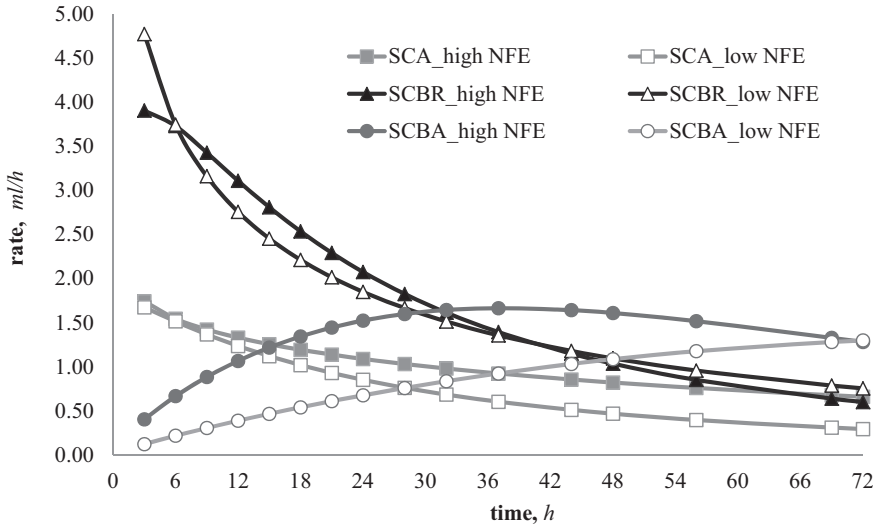

FIGURE 1 In vitro fermentation rate over time for *Saccharomyces cerevisiae* cell wall. SCA, alcoholic extract; SCBA, baker's extract; SCBR, brewer's extract; NFE, nitrogen-free extract

TABLE 3 In vitro end products of *Saccharomyces cerevisiae* in function of production process and NFE concentration

Sample	*n*	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	SCFA	BCFA	NH_3	pH
		mmol/g iOM								mg/L	
Production process effect											
SC-A	8	16.55^{Bb}	4.47^{Bc}	0.553	3.01	0.863	0.117^{Aa}	25.56^{Bb}	0.058^{a}	60.39^{Bc}	6.66^{A}
SC-BR	8	22.05^{Aa}	7.09^{Aa}	0.589	3.04	0.935	0.103^{ABa}	33.81^{Aa}	0.045^{b}	62.92^{Bb}	6.52^{B}
SC-BA	8	18.03^{ABb}	5.68^{Bb}	0.578	2.33	0.937	0.058^{Bb}	27.62^{ABb}	0.055^{ab}	71.60^{Aa}	6.56^{B}
NFE concentration effect											
High	12	17.76	5.84	0.597^{a}	3.02	0.937	0.111^{a}	30.50	0.052	64.17	6.57
Low	12	19.99	5.66	0.549^{b}	2.57	0.886	0.074^{b}	27.50	0.054	65.77	6.57
Interaction		0.089	0.104	0.272	0.501	0.956	0.009	0.142	0.418	0.0006	0.527
RMSE		2.699	0.768	0.0429	1.400	0.148	0.0346	4.536	0.0097	1.965	0.0499

SC-A, *Saccharomyces cerevisiae* alcoholic extract; SC-BR: *Saccharomyces cerevisiae* brewer's extract; SC-BA: *Saccharomyces cerevisiae* bakers extract. NFE, nitrogen-free extract. SCFA, short-chain fatty acids; BCFA, branched chain fatty acids; NH_3, ammonia; RMSE, root mean square error.
Along the column for each effect: A, B = $p < .01$; a, b, c = $p < .05$.

conditions able to guarantee the normal growth and activity of the dog intestinal microbial population. The lower pH values registered fermenting both SC-BA and SC-BR substrates could suggest a higher lactic acid production (Middelbos, Godoy, Fastinger, & Fahey, 2007).

Our findings showed that all tested substrates led to a SCFA production, mainly represented by acetate and propionate, thus suggesting a physiological trend of the in vitro fermentation process. The SCFA production registered after 72 hr of fermentation was higher for all substrates than the data obtained by Calabrò et al. (2013) incubating for 48 hr a spray-dried yeast cell wall (17.89 mmol/g) with dog faecal *inoculum*. On the contrary, similar amounts of SCFA were registered by incubating commercial pre-biotics based on fructooligosaccharides (37 mmol/g) or inulin (39 mmol/g) with dog faeces (Cutrignelli et al., 2009). The slow fermentation process registered in all substrates, and particularly evident in SC-BA, suggests that these substrates are probably fermented more in the distal part of gastrointestinal tract, favouring SCFA production in the colon. Gomes et al. (2008) reported an increase of short-chain fatty acids in faeces from dogs fed with diet supplemented with yeast cell wall. The short-chain fatty acid production is an important indicator of dietary characteristics of carbohydrates source for pet food, and they are the major end products of bacterial fermentation reactions in mammals' colon. The main SCFA (acetate, propionate and butyrate) are rapidly absorbed and then metabolized by the gut epithelium, liver and muscle. Further, it has been reported (Bovera et al., 2010; Mroz, 2005) that SCFA have a trophic effect on the intestinal epithelium, maintaining the mucosal defence barrier against pathogens organisms.

Regarding the BCFA and NH_3 production, compounds derived from the protein degradation by colonic bacteria (Macfarlane, Gibson, Beatty, & Cummings, 1992), the BCFA production registered in this study was higher than that obtained in previous studies by incubating pure fermentable carbohydrates substrates such as FOS and inulin (0.02 and 0.04), but lower than that observed by Calabrò et al. (2013) incubating spray-dried yeast cell wall (1.33), probably for the different crude protein content. The high protein levels of the SC surely affected type and amount of protein catabolites generated through their fermentation in the colon. In particular, the brewer's SC showed the lowest BCFA proportion, according to the lowest crude protein content of this substrate. Indeed, branched chain fatty acids were produced from the metabolism of branched chain amino acids, such as valine, leucine and isoleucine; they can be hydrolysed and fermented to phenols, and biogenic amines (i.e., indole, skatole, 4-ethylphenol, p-cresol) and their production could be related to bacterial autolysis and the fermentation of death bacteria. In this study, the BCFA proportion was not related to the NH_3 production, this is probably due to differences in protein quality in terms of amino acid profile (Davila et al., 2013).

Comparing the substrates used, it seems clear that the production process affects the chemical composition of the yeast cell wall and, consequently, their in vitro fermentation process. In particular, both SC-BR showed very high percentage of OM degradability, gas and SCFA production and a fast fermentation process surely due to the high content of fermentable carbohydrates. On the contrary, both SC-BA yeast cell walls appear to be less degradable and fermentable, probably due to their high content of ether extract. Regarding both SC-A, the high protein content of these substrates could explain the contrasting in vitro results (high degradability and low gas and SCFA production). Indeed, the microbial utilization of protein did not favour directly gas and short-chain fatty acid production (Calabrò et al., 2015; Musco et al., 2017).

5 | CONCLUSION

The findings suggest that the type of production process can significantly influence the nutritional properties of cell wall derived by *S. cerevisiae*, showing significant differences in chemical composition as well as in vitro pathway and fermentation kinetics. Consequently, the use of ingredients with functional characteristics, such as yeast, should be based on chemical composition and in vitro fermentation characteristics.

Considering the volume of gas produced after 72 hr and the fermentation rate, the *S. cerevisiae* brewers extract results highly degradable and fermentable, in terms of amounts of end products of the carbohydrates fermentation and the lower production of compounds of to the protein degradability, suggesting an appropriate fermentation in the large intestine of dog. On the other hand, the moderate fermentation kinetics of *S. cerevisiae* baker's cell wall could be useful to limit the excessive gas production, which always represents an undesirable effect.

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