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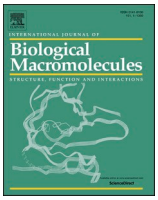

Lignin from sugarcane bagasse as a prebiotic additive for poultry feed

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ABSTRACT

Diet is a crucial factor on health and well-being of livestock animals. Nutritional strengthening with diet formulations is essential to the livestock industry and animal perfor-mance. Searching for valuable feed additives among by-products may promote not only circular economy, but also functional diets. Lignin from sugarcane bagasse was proposed as a potential prebiotic additive for chickens and incorporated at 1 % (w/w) in commercial chicken feed, tested in two feed forms, namely, mash and pellets. Physico-chemical characterization of both feed types with and without lignin was performed. Also, the prebiotic potential for feeds with lignin was assessed by an *in vitro* gastrointestinal model and evaluated the impact on chicken cecal *Lactobacillus* and *Bifidobacterium*. As for the pellet's physical quality, there was a higher cohesion of the pellets with lignin, indicating a higher resistance to breakout and lignin decreases the tendency of the pellets for microbial contamination. Regarding the prebiotic potential, mash feed with lignin showed higher promotion of *Bifidobacterium* in comparison with mash feed without lignin and to pellet feed with lignin. Lignin from sugarcane bagasse has prebiotic potential as additive to chicken feed when supplemented in mash feed diets, presenting itself as a sustainable and eco-friendly alternative to chicken feed additives supplementation.

1. Introduction

Since the second half of the 20th century, livestock industry has developed substantially and one of the reasons that led to this development was the recognition of the importance of animal diet as a crucial factor to influence their health, performance, and well-being. Nutritional strengthening began to be a common prophylactic practice to improve animal gastrointestinal health, with the introduction of antibiotic growth promoters (AGP) in livestock feed to guarantee a high animal productivity performance [1–3]. However, the use of AGP in animal feed was banned in 2006 in Europe and it has been encourage their withdrawal in other parts of the world, due to their adverse effects related to antibiotic resistant microorganisms, increase of the intestinal pathogen's prevalence and susceptibility of the host to them [4–8]. In the last years, there has been an increase in the interest for alternative strategies to in-feed antibiotics (IFA) since livestock animals (specially poultry) are susceptible to a large number of pathogens as a cause of their eating habits and husbandry practices [4,9].

Functional feed additives such as prebiotics (i.e., substrate which is selectively utilized by host microorganisms conferring a health benefit to the host) have been often suggested as alternatives to antibiotics in animal diets. These additives prevent health issues and enhance nutritional quality and average life expectancy as proved by several studies when supplemented in feed matrices [10–12]. Also, prebiotic feed additives play an important role in regulating gastrointestinal tract (GIT) microbiota, which in turn play an essential role in animal's general health and productive performance [13–15]. The most common prebiotic feed additives studied and used in poultry diets are inulin, fructo-oligosaccharides (FOS) and mannan-oligosaccharides (MOS) [16,17]. Prebiotics positively stimulate the proliferation and activity of beneficial bacteria in poultry intestinal microbiota, such as *Lactobacillus* and *Bifidobacterium* which are related to the production of short-chain fatty acids (SCFA), that have important roles, such as regulation of gut internal environment, improvement of the immune system, inhibition of acid-sensitive pathogens growth, among others [12,13,18–20]. Most of the studies related with poultry microbiota and/or prebiotic potential

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are focused mainly on the cecal microbiota. The cecum is the most microbial densely populated and diverse section in the poultry GIT, with a digesta passage time between 12 and 24 h, and the main region for bacterial anaerobic fermentation and pathogens' colonization [13,20]. The supplementation of functional ingredients in feed matrices must consider and evaluate the ingredient-feed matrix interaction(s), as these interactions can be a important factors in the selection of functional ingredients for incorporation in a specific feed matrix to maximize its potential when fermented by the intestinal microbiota [21,22].

Lignin is one of the main components of plant biomass and the source of renewable aromatic structures on the planet. It is a complex phenolic macromolecule formed by radical polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols [23]. The arrangement of the monomers creates a wide range of structures with different functional groups and unique properties. Recent studies have highlighted the potential of lignin in energy production [24], biorefinery [25], materials science [26,27], and biotechnology [28]. In energy production, the aromatic groups present in the lignin structure make it a suitable feedstock to produce biofuels like bioethanol and biodiesel. In biorefinery, lignin can be used to produce high-value chemicals like vanillin or as a source of carbon fiber used in the aerospace and automotive industries [29]. Lignin is also an attractive alternative to petroleum-based materials due to its unique capability of crosslinking with other compounds, renewability, and abundance [30]. Applications such as a natural binder, coating and adhesive in composites opened avenues to exploit its flexibility and provide adjustable mechanical and thermal properties to materials with improved durability in the construction industry [30]. Lignin is also of great interest in biomedicine and biotechnology, where its low solubility, pH-responsive chemistry, UV-blocking effect, and high stability provides remarkable applications as drug delivery carriers and tissue regeneration [27]. The leverage of the abundance of the hydroxyl groups in the structure of lignin makes it a good source of phenolic compounds with attractive antioxidant and antimicrobial properties to be used in health, cosmetics, human and animal nutrition [31]. In respect to the animal nutrition field, lignin has been attracting the interest of scientific community as a prebiotic additive [32,33]. In contrast to native lignin, purified lignin does not represent a barrier to digestion in monogastric animals, exerting health benefits and could potentially be considered as a natural feed additive [34]. Purified lignin is rich in low molecular weight mono-phenolic fragments (e.g., carvacrol and cinnamic acid) which have been shown to be effective antioxidants and food preservatives to inhibit microbial growth and can be beneficial to favor *Lactobacillus* and *Bifidobacterium* balance, to limit the colonization of intestinal pathogens, contributing to different biological characteristics of native lignin [34–37]. Alcell lignin (obtained by organosolv process) was reported to show prebiotic effect in poultry by promoting the growth of *Lactobacillus* and *Bifidobacterium* [38,39]. The interest of purified lignin as a prebiotic feed additive is due to the fact that can be obtained from undervalued agro-industrial residues sources, with no direct applicability, enabling a sustainable approach within a circular economy framework [32,40–42]. Sugarcane is an example of a plant crop that produce a significant number of by-products such as sugarcane bagasse (SCB), from which lignin can be extracted.

The feed manufacturing process is also important for the additives incorporation as it affects the nutritional value and stability of feed products. Several studies have been focused on the importance to evaluate different feed forms regarding its impacts on broilers microbiota to comprehend and establish a relation between the feed form (e.g., mash and pellets) and nutrient availability [43–45]. Different feeding forms have its own advantages and disadvantages, for example, mash is associated with better feed conversion efficiency, less death loss and cheaper than other forms (e.g. crumbles, pellets), while pellet is associated with reduction on feed wastage, higher growth rate, and increased feed intake [46,47]. Nevertheless, it is crucial to access if the pellet binder addition improves the physical pellet quality without compromising nutrient availability [43].

The aim of this study was to assess the potential of lignin from SCB as a prebiotic feed additive. This experiment used alkaline lignin and 1 % (w/w) to be incorporated into two forms of chicken feed (with the same composition): mash and pellet. The physico-chemical properties and the influence of the feeding forms on the poultry cecal microbiota were investigated.

2. Materials and methods

2.1. Lignin extraction and characterization

Lignin extraction and characterization was previously reported by Costa and co-workers [48]. Briefly, SCB was air-dried overnight in an oven at 40 °C, milled (Retsch model SM100, Haan, Germany) and sieved (Retsch AS 200 basic, Haan, Germany). Then, SCB was mixed with 2 % NaOH (LabChem, Zelienople, PA, USA) solution (g/g) in a liquid to solid (L/S) ratio of 15 g/g. Experiments were carried out in a water bath at 90 °C for 1 h. After the extraction, solid (pre-treated biomass) and liquid (alkaline liquor) phases were recovered by vacuum filtration. The liquid phase was acidified with H_2SO_4 (Honeywell Fluka, Seelze, Germany) for lignin precipitation. The precipitate was recovered by centrifugation, washed with deionized water and oven dried at 60 °C. Regarding lignin characterization, the moisture, dry matter, and ash content were gravimetrically determined at 105 °C (Venti-Line Prime, VWR, Carnaxide, Portugal) and 550 °C (Nabertherm L15/12/B410, Hagen, Germany), respectively, until constant weight. The crucibles were previously calcined overnight at 550 °C. All samples were analyzed at least in duplicate.

2.2. Chicken feed formulation

The chicken feed was formulated by milling the commercial chicken feed kindly supplied by Sorgal S.A. (Aveiro, Portugal) in a mill (Kenwood multipro compact, Havant, UK) and the lignin was incorporated homogeneously by dry mixing (180 s) to obtain fine particles. This mixture was used for further analysis. To obtain the pellets, water (at a maximum of 10 % (w/w)) was added to the mixture and subjected to a cold granulation process (process temperature did not exceed 40 °C) using an extruder (Häussler Nudelmaschine PN 100, Altheim, Germany) and dried in the air. The chicken feed - used as control (named as baseline) and for lignin incorporation - is generally composed of corn, peeled and roasted soybean bagasse obtained by extraction, roasted soybean seeds, rapeseed bagasse, poultry fat, calcium carbonate, monobasic calcium phosphate, sodium chloride, and sodium bicarbonate. The designation of the different forms of chicken feed was determined as mash baseline feed (MBF), MBF supplemented with 1 % (w/w) SCB lignin (MBF +1 % lignin), pellet baseline feed (PBF) and PBF supplemented with 1 % (w/w) lignin (PBF +1 % lignin). Lignin was tested at 1 % (w/w) as it is a prebiotic percentage commonly used in feed matrices (~1–3 %) [16,17,19,49].

2.3. Chicken feed characterization

2.3.1. Particle size of mash particles

The MBF was characterized for its particle diameter through an accumulative granulometric analysis (integral method) using an electromagnetic stirring (Retsch analytical sieve shaker model AS200 basic, Haan, Germany) (mash sizes from 100 to 800 μm), and the exact weight of each sieve was recorded. Then, MBF samples were shaken and washed 3 times for 3 min under a continuous tape water flow. The sieves were subsequently placed in a dry-force oven for 24 h at 105 °C. The dry weight of particles retained by each sieve was expressed as a proportion of the total powder recovered and calculated by dividing by the initial total weight of the sample.

2.3.2. Moisture, dry matter and ash

The moisture, dry matter and ash contents were gravimetrically determined as previously described in Section 2.1. All samples were analyzed at least in duplicate.

2.3.3. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) analysis

ATR-FTIR analysis was performed using a FT-IR spectrometer (Perkin Elmer, Waltham, USA). Samples were placed until the ATR crystal was completely covered. The samples were scanned from 550 cm^{-1} to 4000 cm^{-1}, with 16 scans at a nominal resolution of 4 cm^{-1}. For all spectral manipulation the Perkin Elmer FTIR Software (Perkin Elmer, Waltham, USA) was used.

2.3.4. Dynamic scanning calorimetry (DSC) analysis

The thermal properties were assessed by differential scanning calorimetry (DSC 204 *Phoenix*, Netzsch, Waldkraiburg, Germany). Samples weighing ca. 1–5 mg were sealed in 40 μL aluminum pans and heating and cooling runs were performed from 0 °C to 500 °C at a heating rate of 10 °C/min under a constant nitrogen flow (40 mL/min). An empty pan was used as a reference. Indium (purity >99.95 %; Fluka, Buchs, Switzerland) was employed for calibration purposes. DSC thermograms were recorded using Proteus analysis software (Netzsch, Waldkraiburg, Germany).

2.3.5. Water and oil absorption capacity

To quantify the water and oil absorption capacity, distilled water and linseed oil were used. Briefly, 1 g of the sample was mixed with 10 mL of water or oil, incubate at 60 °C for 30 min and centrifuged for 15 min at 1000 rpm. The value obtained was expressed as % water and oil bound per gram of the sample and was calculated according with the following Eq. (1).

$$\text{WAC or OAC} = \frac{\text{final sample mass (g)} - \text{initial sample mass (g)}}{\text{initial sample mass (g)}} \quad (1)$$

2.3.6. Determination of water activity

The water activity (a_w) of the samples was determined at 25 °C using a Rotronic HygroLab laboratory analyzer (Rotronic, Bassersdorf, Switzerland). After calibration and equilibration, the water activity value was recorded.

2.3.7. Apparent bulk density

The apparent bulk density of the pellets was determined by weighing around 1 g of sample and putting in a 25 mL glass cylindrical with water (20 mL of water) and tapping 20 to 30 times, until no noticeable change in volume. The apparent bulk density was determined as the weight per unit volume of the sample.

2.3.8. Texturometer analysis

The hardness and cohesion of the pellets was determined using a TA-XT2 Plus texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a 10 kg load cell. A cylindrical probe with 36 mm diameter was used. A compression test was selected. The pellets were deformed 60 % of their total height. Twenty measurements were taken for each different pellet composition. The pellets dimensions analyzed were similar for both different combinations ranging between 4 and 6 mm of length. The surface tensile strength was calculated from the measured crushing force F and the pellet mean radius R by using the Eq. (2) [50]:

$$\sigma f = \frac{0.4F}{\pi R^2} \quad (2)$$

2.4. Prebiotic assessment

2.4.1. Cecal inoculum preparation

Cecal content was obtained from 18 carcasses of 38-days broiler chickens (Ross 308) with an average weight of 1.90 ± 0.19 kg at Savinor slaughterhouse (Covelas, Portugal), fed with an antibiotic-free maize and soybean diet. Prior to their slaughter, the animals underwent a fasting period of 8–10 h. The animals were subjected to electrical stunning, and the cecum removed, clipped on both sides with a string, properly identified and stored in a clean tamper proof specimen 1 L containers (Sigma, St. Louis, MO, USA). The containers were placed in an Oxoid™ AnaeroJar™ 2.5 L (Thermo Fischer Scientific, Waltham, CA, USA) containing an Oxoid™ AnaeroGen™ 2.5 L sachet (Thermo Fischer Scientific, Waltham, CA, USA), closed, and only opened inside an anaerobic cabinet (nitrogen 80 %, carbon dioxide 10 %, and hydrogen 10 %), Whitley A35 workstation (Don Whitley Scientific, Bingley, UK), within 2 h of collection.

Under anaerobic conditions, the cecal content for each cecum was squeezed into an empty pre-weighted tamper proof specimen 1 L container, weighted and the cecal pool diluted at 10 % (w/w) with 0.1 M phosphate-buffered saline (PBS) (Thermo Fischer Scientific, Waltham, MA, USA) solution with 5 % (v/v) dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). The cecal slurry was first homogenized manually and further mechanically with a Mixwel® laboratory blender (Alliance Bio Expertise, Guipry, France) for 2 min at 460 paddles beats/min and aliquots of 15 mL cecal inoculum were prepared. The cecal inoculum were storage at −80 °C (**Appendix A - Table S1**).

2.4.2. Chicken gastrointestinal tract (GIT) simulation model

An *in vitro* GIT simulation model adapted from [51–53] was carried out with modifications described in [54]. Four different feeds were subjected to this protocol: 1) MBF, MBF supplemented with 1 % (w/w) FOS (Megazyme, Bray, Ireland), MBF supplemented with 1 % (w/w) lignin and PBF supplemented with 1 % (w/w) lignin. For the oral phase, 208 μL of 1 mM $CaCl_2$ (Merck KGaA, Darmstadt, Germany) (pH 4.5–5.5) was added to 4 g of the testing condition in a 100 mL erlenmeyer. The feed mixture was place at 41 °C for 40 min without agitation. After oral digestion, 25 mL of chicken gizzard digestive juice (1 M NaCl ((Honeywell Fluka, Seelze, Germany), 10 g L^{-1} pepsin from porcine gastric mucose powder- P7000 (Sigma, St. Louis, USA), adjusted to pH 2.0 ± 0.1 with 6 M HCl (Honeywell Fluka, Seelze, Germany) was added to the feed mixture and incubated at 41 °C, 120 rpm for 1 h, to mimic gastric digestion. Finally, to initiate the intestinal digestion phase, the feed mixture pH was adjusted to 6.2 using a 1 M $NaHCO_3$ (Panreac, Barcelona, Spain) solution and 2.52 mL of the volume of each condition discarded and replaced by the same volume with intestinal digestive juice (3.5 % (w/v) bile extract B8631 (Sigma, St. Louis, USA) and 0.35 % (w/v) pancreatin from porcine pancreas- P7545 (Sigma, St. Louis, USA) in deionized water, pH = 6.80 ± 0.04). The mixture was incubated at 41 °C, 120 rpm for 3 h. The last stage of the GIT is the intestinal absorption phase, in which the simulated feed digesta was transferred into a 1 kDa dialysis membrane (Spectrum, New Brunswick, USA) clipped on both edges, submerged in a 10 mM NaCl solution, and left overnight, stirred at 150 rpm and room temperature. The retained substrates were freeze-dried in an Alpha 2–4 LSC plus model (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and used as feed substrates in the in vitro batch culture fermentation system.

Sterile stirred batch culture fermentation vessels of 300 mL were set up and aseptically filled with 135 mL sterile basal nutrient medium according to [55] and gassed overnight with O_2-free N_2, with continuous agitation. Each condition was assessed in duplicate, the substrates added aseptically (by flaming the entry/sampling port) and fermented by the cecal inoculums. Vessels (1) and (2) contained 1 % (w/v) of the digested MBF; vessels (3) and (4) 1 % (w/v) of the digested MBF supplemented with 1 % (w/w) FOS (positive control); vessels (5) and (6) digested MBF supplemented with 1 % (w/w) lignin, vessels (7) and (8) digested PBF supplemented with 1 % (w/w) lignin; and vessels (9) and (10) the inoculum control (IC) with no substrate added (negative control). Once the substrates were properly mixed with the basal media, each vessel was inoculated with 15 mL of frozen cecal slurry. For the frozen

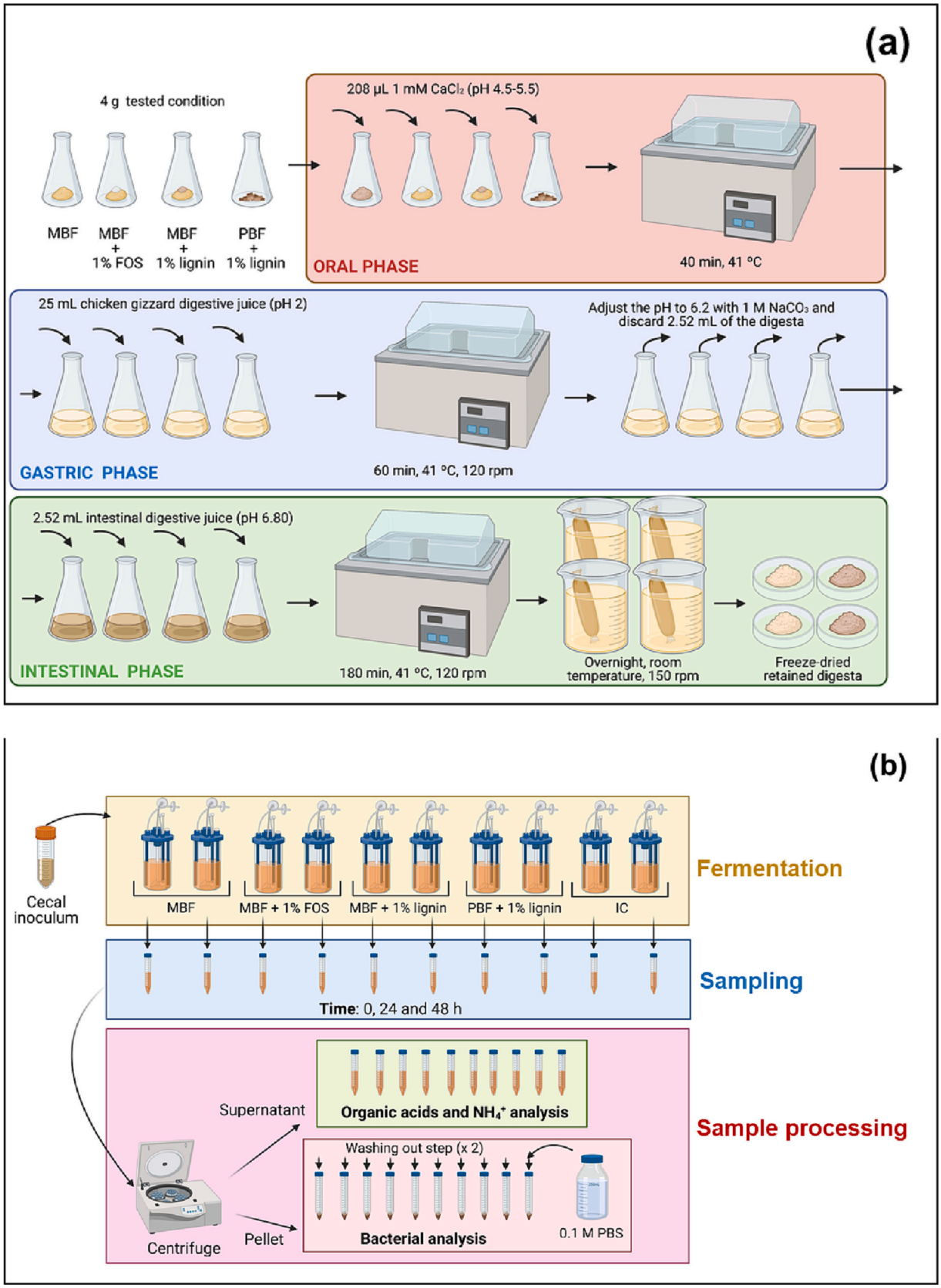


Fig. 1. (a) Chicken gastrointestinal tract (GIT) simulation assay work fluxogram and (b) sample processing performed for this study. The Figure was created with BioRender.com.

Table 1
Group-specific primers based on 16S rDNA sequences to profile cecal fermentation samples.

Primer	Target organisms	Genomic DNA standard	Media borth	Media agar	Incubation conditions	Sequence (5′-3′)	Amplicon size (bp)	Reference
Lac	*Lactobacillus*	*Lactobacillus gasseri* DSM 20077	MRSB[a]+0.1 % (w/v) cysteine	MRSA[a]+ 0.1 % (w/v) cysteine	Anaerobic 37 °C 2 days	**F**[a]: CACCGCTACACATGGAG **R**[a]: AGCAGTAGGGAATCTTCCA	341	[58,59]
Bif	*Bifidobacterium*	*Bifidobacterium animalis* ssp. *lactis* BB-12 DSM 15954				**F**[a]: CGCGTCYGGTGTGAAAG **R**[a]: CCCCACATCCAGCATCCA	244	[60]

[a] Legend: F- foward; MRSB/A- de Man, de Rogosa and Sharpe Broth/Agar (Biokar Diagnostics, Allonne, France); R- Reverse.

inoculums were previously submitted to a two-DMSO wash-out cycles according to [56], to eliminate the DMSO, used as cryo-preservative during storage. A FerMac 260 pH controller (Electrolab Biotech Ltd., Gloucestershire, UK) was used to maintain the pH between 6.0 and 7.0 (the pH of the chicken cecum) in each vessel [57], and temperature was kept at 41 °C with the help of a water bath. Batch fermentations were run for 48 h and samples (10 mL) were taken aseptically from each vessel, at 0, 6, 24, 30, and 48 h for short chain fatty acids (SCFA) and lactate analysis by HPLC, ammonium (NH_4^+) concentration measurement, using an ion-selective electrode 9663 of ammonium (Hach, Colorado, USA) and bacterial enumeration by quantitative polymerase chain reaction (qPCR). Immediately after collection, samples were placed in ice to stop the fermentation, centrifuged at 4 °C, 4696 x*g* for 5 min, and the supernatant collected for HPLC analysis. The pellet was resuspended in 10 mL of a 0.1 M PBS solution, vortex, and centrifuged (as described previously). The supernatant was discarded, and the washing cycle repeated once more time. Pellets from 0, 24 and 48 h were stored at −20 °C until qPCR analysis (Fig. 1).

2.4.3. Bacterial enumeration by culture independent methods

The DNA samples from sampling time 0, 24 and 48 h of cecal fermentation were extracted. The total DNA was extracted from the pellet obtained of each sapling time using PureLink™ Microbiome DNA Purfication Kit (Invitrogen, Massachusetts, USA) according to the DNA extraction protocol provided by the manufacturer. The quantification of DNA concentration was measured by a Qubit 4 fluorometer following the Qubit ® dsDNA HS assay kit protocol (Invitrogen, Massachusetts, USA). The final DNA concentration of each sample was adjusted to 10 ng/μL. The targeted groups, primer sequences, amplicon sizes and literature references are depicted in Table 1. Conditions for qPCR reactions were prepared to a final volume of 10 μL, containing of 1 x NZYSpeedy qPCR Green Master Mix (NZYTech, Lisbon Portugal), 1 μM of each primer (forward and reverse), 2 μL of DNase/RNase-free water and 1 μL of template DNA. In the negative control 1 μL of DNase/RNase-free water was use instead of template DNA. The cycling conditions were 95 °C for 10 min (polymerase activation), 95 °C for 15 s (denaturation), then 40 cycles of 60 °C for 1 min (annealing). Additionally, analysis of melting curve was performed. For the bacterial quantification, DNA of bacterial monocultures were used to create standard calibration curves (Table 1). Briefly, for each set of primers five decimal dilutions of bacterial DNA were prepared to plot a standard calibration curve, which correlates the cycle threshold (Ct) values and log colony-forming unit (CFU) per mL.

2.4.4. Determination of organic acids produced during in vitro fermentation

The supernatants collected after centrifugation were filtered (0.22 μm) and directly analyzed by HPLC in duplicates, as described in [56] with slight modifications. Conditions for the HPLC system consisted in an Agilent 1260 II series HPLC instrument (Agilent, Santa Clara, CA, USA) with a refractive index (RI) detector and diode array detector (DAD) at 220 nm, and an ion-exclusion Aminex HPX-87H column (Biorad, Hercules, CA, USA) operated at 50 °C. A 5 mM sulfuric acid (H_2SO_4) ((Honeywell Fluka, Seelze, Germany) mobile phase was used at a flow rate of 0.6 mL/min, with a running time of 40 min and injection volume of 10 μL. Lactate, acetate, propionate, butyrate, isobutyrate and isovalerate (Sigma, St. Louis, MO, USA) were identified and quantified using their corresponding calibration curves (2 mM to 80 mM).

Table 2
Lignin from sugarcane bagasse (SCB) moisture, dry matter, ash, total sugars and purity (%, means±SD).

Parameters (%)	
Moisture	0.74 ± 0.05
Dry matter	99.26 ± 0.11
Ash	2.05 ± 0.01
Sugars	1.68 ± 0.11
Purity	94.9 ± 0.10

2.4.5. Measurement of total ammonia nitrogen concentration

An ion-selective electrode 9663, at constant temperature (room temperature, 20 °C) and pH (6.0–7.0), was used for the measurement of ammonium concentration. According to manufacture instructions, 300 μL of 1 M $MgSO_4$ (Honeywell Fluka, Seelze, Germany) (ionic strength adjuster) was added to 3 mL of the supernatants collected after centrifugation and readings carried out in duplicate using an electrode. For quantification, a standard calibration curve of NH_4Cl (Mettler Toledo, Urdorf, Switzerland) (2 mM to 55 mM) was used. Total ammonia nitrogen concentration was calculated according to the equation below (Eq. (3)) [61]:

$$\frac{\left[NH_4^+\right]}{\left[NH_3 + NH_4^+\right]} = 1 - \frac{1}{1 + 10^{pKa-pH}} \quad (3)$$

where, $[NH_4^+]$ is the ammonium ion concentration, $[NH_3 + NH_4^+]$ is the total ammonia nitrogen concentration, and pKa is the acid dissociation constant that can be expressed as a function of temperature (T) using the following Eq. (4) [61]:

$$\mathrm{pKa} = 4 \times 10^{-8} T^3 + 9 \times 10^{-5} T^2 - 0.0356T + 10.072 \quad (4)$$

2.4.6. Statistical analysis

Data are presented as the mean ± standard deviation of at least two independent assays. Statistical evaluation of the data between two groups was performed by a student's *t*-test and between more groups by one-way analysis of variance (ANOVA) test using GraphPad Prism version 8.0. Tukey's HSD test multiple comparison test was carried out to compare the significance of the different groups. The statistical significance was recorded as the *p*-value $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. Data's normality of the prebiotic assessment was evaluated using Shapiro–Wilk's test. As the samples followed normal distribution, means were compared considering a 95 % confidence interval, using one-way ANOVA coupled with Tukey's post-hoc test.

3. Results and discussion

3.1. Lignin characterization

The properties of lignin regarding the purity, moisture, dry matter,

Table 3
Moisture, dry matter and ash content in the chicken feed forms.

Feed form	Moisture	Dry matter	Ash
MBF	1.39 ± 0.15	98.61 ± 0.31	5.28 ± 0.05
MBF + 1 % lignin	1.44 ± 0.0	98.56 ± 0.14	5.17 ± 0.08[a]**
PBF	7.40 ± 0.01[a, b, c]****	92.60 ± 0.01 [a, b]****, [c]***	5.40 ± 0.03
PBF + 1 % lignin	4.49 ± 0.02[a, b]****	95.51 ± 0.0 [a; b]***	5.36 ± 0.04

The results are expressed as the mean, % ± SD of three independent assays (a - different from the MBF; b - different from the MBF + 1 % lignin; c - different from the PBF + 1 % lignin; * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$).

ashes, and sugars are depicted in Table 2. SCB lignin presented high purity (94.9 ± 0.1 %) and a particle size <40 μm. As already mentioned, lignin from SCB has been rarely exploited to animal nutrition, however other alkaline lignins from different sources have been suggested for feedstock for biomass pellets. According to those studies, the amount of NaOH used in the extraction process can affect biomass characteristics, specifically, it can negatively influence the ash content [62]. Overall, the parameters of this lignin from SCB seem to be comparable and acceptable for food nutrition purposes.

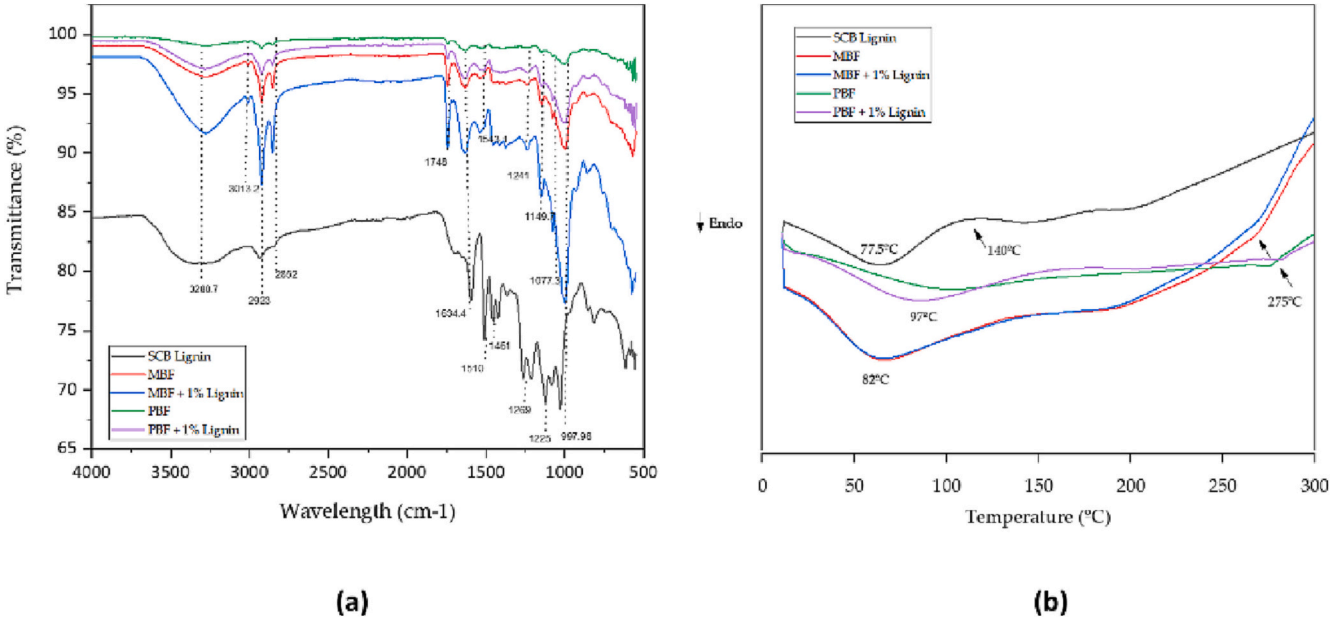
3.2. Effect of lignin-derived additive on mash and pellets characteristics

The effect of SCB lignin at 1 % (w/w) on mash and pellets characteristics was evaluated for several physico-chemical properties, that in turn can affect the absorption and digestion of nutrients. Regarding the particle size of the mash, most of the particles obtained after grinding (> 80 %) showed a diameter between 160 and 800 μm (data not shown). The incorporation did not affect the initial particle size and both mashes, MBF and MBF + 1 % lignin guaranteed an optimum particle size, which is around 600 and 900 μm. It has been shown that grain particle size is more critical in mash diets, than in pelleted or crumble diets [44]. The pellets were also compared, and dimensions of the PBF +1 % lignin revealed to be equal from the PBF, being around 3 to 6 mm of length. The granulometry of the feed is a very important factor which influences the performance and availability of nutrients and additives for a correct absorption and digestion, which will be traduced in a better performance and chicken's health [63].

The moisture, dry matter and ash content of the mash and the pellets were assessed, and the results are presented in Table 3. Higher moisture values were detected in the pellets, presenting significant differences from the MBF conditions ($p < 0.0001$). This difference can be attributed to the water incorporated into the formulation of the pellets, essential for an adequate material viscosity. As so, the dry matter is higher for the physical mixtures, which somehow it is translated by the content on fiber, proteins minerals and carbohydrates. There is a difference between the baseline feed form, namely the mash and pellets ($p < 0.0001$), and the addition of lignin was only significant for the pellet form ($p < 0.001$). Regarding the ash content, the mash form was the only condition showing a significant difference when supplemented with lignin ($p < 0.05$). The ash composition is reported to be high in minerals, such as calcium, phosphorous, potassium and magnesium. From the nutraceutical point of view, ash is not digestible by animals, however, in a suitable concentration it is essential to their health. The normal values of ash content in feed is between 5 and 8 % to promote a good balance in the nutrients availability and energy utilization on animals [64]. According to our results (Table 3), lignin seems to not interfere with this content compared with the baseline feed.

3.3. Chemical and thermal profiles

The chemical composition of the mash and pellets was evaluated by ATR-FTIR to assess the pelleting and the effects of lignin incorporation at 1 % (w/w). According to the FTIR spectra depicted in Fig. 2a, the baseline chicken feed in both forms, MBF (red line) and PBF (green line) did not revealed differences in ATR-FTIR spectra. Due to its composition, the feed presents a complex mixture comprehending several nutrients and supplements. Nevertheless, considering the proximate composition and the main ingredients of the diet, some fingerprints can be highlighted, namely the contributions assigned to lipids (3013 cm^{-1}, 1748 cm^{-1}), proteins (1634 cm^{-1}, 1543 cm^{-1}), carbohydrates (1149 cm^{-1}, 1077 cm^{-1}, 1000 cm^{-1} and 997 cm^{-1}), fatty acids (1241 cm^{-1}1149 cm^{-1}), amides I (region at 1569–1730 cm^{-1}) and amide II (region at 1480–1569 cm^{-1}) molecules. The broad band between 3650 and 3050 cm^{-1} can be associated with the overlap of O—H and N—H stretching of



(a) (b)

Fig. 2. (a) FTIR spectrum and (b) thermograms of chicken meal feed in mash and pellets feeds, without and with lignin incorporation at 1 % (w/w). The results were depicted for MBF - Mash baseline feed; MBF + 1 % lignin - Mash feed with 1 % (w/w) lignin; PBF - Pellet baseline feed; PBF + 1 % lignin - Pellet feed with 1 % (w/w) SCB lignin and SCB lignin.

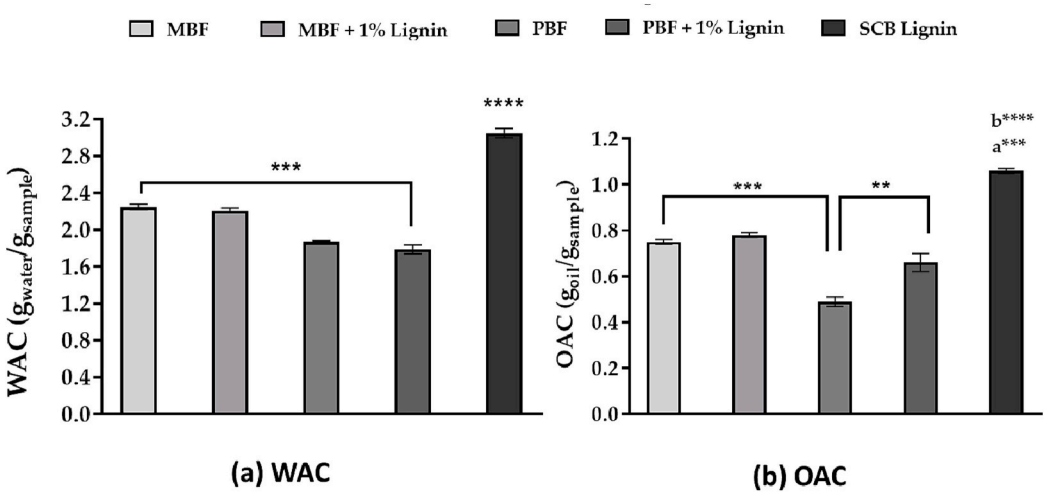


Fig. 3. (a) Water Absorption Capacity (WAC) and (b) Oil Absorption Capacity (OAC) of the different chicken feed forms, without and with 1 % (w/w) of SCB lignin. The results are expressed in g water or oil/g sample. All the results are expressed as the mean ± SD of two independent assays. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; a - different from both mash conditions; b - different from both pellet conditions (ANOVA, Tukey HSD). MBF - Mash baseline feed; MBF + 1 % lignin - Mash feed with 1 % (w/w) lignin; PBF - Pellet baseline feed; PBF + 1 % lignin - Pellet feed with 1 % (w/w) SCB lignin.

amide I vibrations very common on proteins and amino acids based samples. Concerning the pelleting, there is no evidence that chemical composition of feed suffers any alteration during this process. Regarding the incorporation of 1 % of lignin, it was observed no significant FTIR spectral differences for both feed forms, mash, and pellets. The spectra were completely superimposable for both supplemented and non-supplemented diets, with no evidence for the lignin typical peaks, namely those at 1510 and 1461 cm^{-1}, associated with the usual abundance of aromatic rings and aliphatic structures present in lignin structure. As observed in the lignin ATR-FTIR spectrum (Fig. 2(a) black line), it is visible the characteristic peaks of the syringyl and guaiacyl units, namely at 1269 and 1225 cm^{-1} [65], respectively. In both supplemented feed forms spectra, those peaks are not evidenced, which can be attributed to the low content of lignin incorporated. The thermal behavior of both feed forms was performed by DSC analysis in a range of temperatures from 0 to 300 °C (Fig. 2(b)). Differential Scanning Calorimetry obtained for the diet mash and pellets with and without lignin at 1 % (w/w) were superimposable, revealing that 1 % (w/w) lignin incorporation did not induce any significant chemical changes in the thermal profile of both feed forms (Fig. 2(b)). Regarding the feed mash and pellets, it was observed a slight shift of the first thermal event from 82 °C to 97 °C, mainly associated to the loss of water until 100 °C. This peak shift can be explained by the higher content of water present in the pellets than in the mash, necessary to the extrusion process. The feed diets start to degrade at around 250–275 °C, mainly associated with the soybean meal feed composition rich in soy fibres (≈275 °C). The glass transition observed in lignin at about 140 °C, it was not observed in both feed forms, mash, and pellets, which may be related to the low percentage of incorporation.

3.4. Effect of lignin additive on water and oil absorption capacities

The water absorption capacity (WAC) and the oil absorption capacity (OAC) are important functional properties in animal feed that may influence flavor, texture, softness, and palatability [66]. The evaluation of these parameters on the chicken feed form with and without lignin from SCB at 1 % (w/w) is depicted on Fig. 3. SCB Lignin alone revealed higher values than in formulation for both functional properties compared to the other conditions ($p < 0.001$ and $p < 0.0001$, Fig. 3). Other studies previously reported that raw lignin biomass can readily absorb moisture due to the presence of hydroxyl groups (single bond OH) that form hydrogen bonds and retain additional water [67,68]. However, when in moisture, the hydroxyl groups present in lignin may interact with other feed components, such as vitamins and nutrients, leading to a decrease in their number and the formation of nonpolar unsaturated structures [69]. This effect can reduce the lignin's capacity to absorb water.

Table 4
Water activity (a_w) of different chicken feed forms tested, without and with 1 % (*w*/w) SCB lignin.

Feed form	a_w	Temperature (°C)
MBF	0.617 ± 0.001[a]	27.125 ± 0.092
MBF + 1 % lignin	0.611 ± 0.001[a]	27.180 ± 0.085
PBF	0.637 ± 0.004[a]	23.880 ± 0.115
PBF + 1 % lignin	0.507 ± 0.002	23.633 ± 0.058
SCB lignin	0.537 ± 0.005	27.345 ± 0.007

The results are expressed as the mean ± SD of three independent assays (a ****$p < 0.0001$ different from the pellets with 1 % (w/w) lignin).

Similarly, the OAC can also be affected by changes in the chemical configuration and composition of lignin.

Concerning the results for both parameters, OAC and WAC, significant differences ($p < 0.001$) were found between mash and pellets. This can be attributed to the mash ability to absorb a higher amount amount of water or oil associated with the higher surface area and higher packing density promoted by the lower particle sizes of the mash as compared to the pellets [70]. Considering the effect of lignin on each feed form, it seems to not affect particularly the values of WAC (Fig. 3 (a)). The WAC is an important parameter that will determine the correct digestion of the feed in the gastrointestinal tract (GIT), and lignin incorporation seems to not significantly affect in both feed forms. Concerning the MBF, the OAC slightly decreased with the incorporation of lignin, being the value of OAC of the MBF of 0.78 % and MBF + 1 % lignin was 0.75 % (Fig. 3(b)). For the PBF, the OAC increased significantly from 0.49 % to 0.66 % (PBF +1 % lignin) ($p < 0.01$). Despite a higher absorption capacity, it is not expected to have a significant impact on the pellet's lipids digestibility. Herein, it is possible to observe that lignin only revealed that raise in the pellet form, which indicates the binding capacity of the nutrients within the pellets. In fact, OAC has been attributed to the physical entrapment of oil within proteins and noncovalent bonds such as hydrophobic, electrostatic, and hydrogen bonding as forces involved in lipid–protein interactions [71]. As so, the OAC can be translated by the capacity of an ingredient act as a binder with fat during food processing, indicating this capacity for lignin in the pellet feed form.

3.5. Lignin potential for protection of feed from microbial contamination

According to the results depicted on Table 4, the incorporation of 1 % lignin in the MBF did not affect the a_w of the mixture. For both mash samples, the a_w was around 0.61. Regarding the pellets, it was possible to observe a positive significant effect of lignin in the final product since

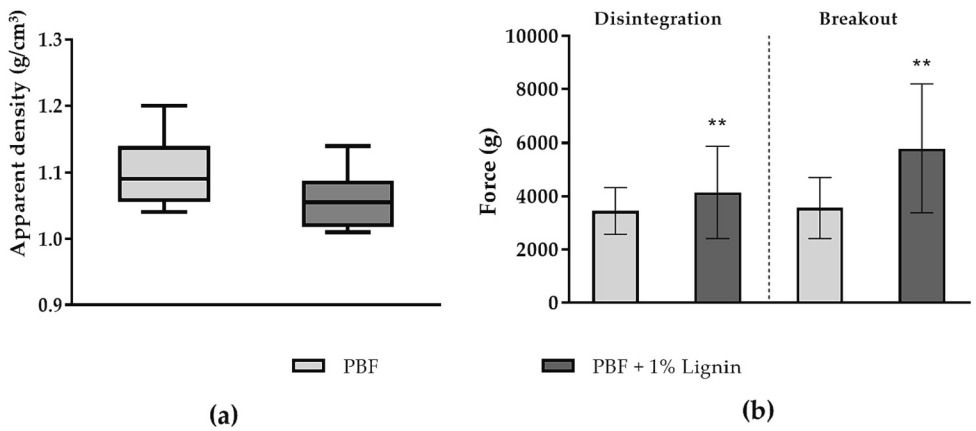


Fig. 4. s(a) Apparent density determination (g/cm^3, mean ± SD, $n = 6$) and (b) mechanical behavior of the pellets translated by the force (g, mean ± SD, $n = 20$) necessary to disintegrate and break the standard pellets and the pellets with 1 % (*w*/w) lignin. ** $p < 0.05$ (unpaired *t*-test). PBF - Pellet baseline feed; PBF + 1 % lignin - Pellet feed with 1 % (w/w) SCB lignin.

Table 5
The resistance to deformation of the PBF and the PBF + 1 % lignin obtained on the mechanical behavior assessment.

Sample	σf (N/mm)
PBF	59.68 ± 15.14
PBF + 1 % lignin	61.65 ± 19.83

it presented a lower a_w (0.507) as compared to the PBF (0.637) ($p < 0.0001$). Minimum a_w values are desirable to avoid growth and toxin production by microorganisms in food. As observed, lignin generated a lower risk of contaminations and possible chemical reactions that can compromise the stability and viability of the pellets chicken diet, since lower free water is available to promote those events. The reduction on the a_w can be a result of the physical binding of water to lignin by hydrogen bonding van der Waal forces. According to the FDA [72], a finished product should present a_w values lower than 0.85 which, together with other parameters, contribute to achieve the commercial sterility of the product being able to minimize the growth of most microorganisms (bacteria, mold, fungi).

3.6. Influence of lignin on the pellet's physical parameters

The physical quality of the chicken feed pellets can be evaluated using pellet density and pellet hardness parameters. These parameters are very important since the final pellets must be homogeneous and present the suitable hardness to be accepted by the animals. The results of the apparent density determination of the PBF and the PBF + 1 % lignin are depicted in Fig. 4(a). The PBF depicted an apparent density of 1.10 g/cm^3 and the density of the PBF + 1 % lignin was 1.06 g/cm^3. The results showed that the addition of lignin to formulation did not significantly affect the density of the final product – pellets chicken diet. Usually, the density of this feed form for poultry can vary from 0.61 to 1.24 g/cm^3 without causing any disturbance to digestion of the chicken [73], and these results in accordance with those recommended values. The influence of lignin on the pellet hardness was measured to assess its impact in the physical integrity of the pellets (Fig. 4(b) and Table 5). Both pellets' compositions were compressed revealing two different peaks: a primary peak characterized by a disintegration of the pellet followed by a second peak representing the moment at which the pellets are compressed until breakout. According to Fig. 4(b), lignin revealed to not considerably affect the hardness of the pellets since the Force for the disintegration of the PBF was 3445.88 g and the PBF + 1 % lignin was

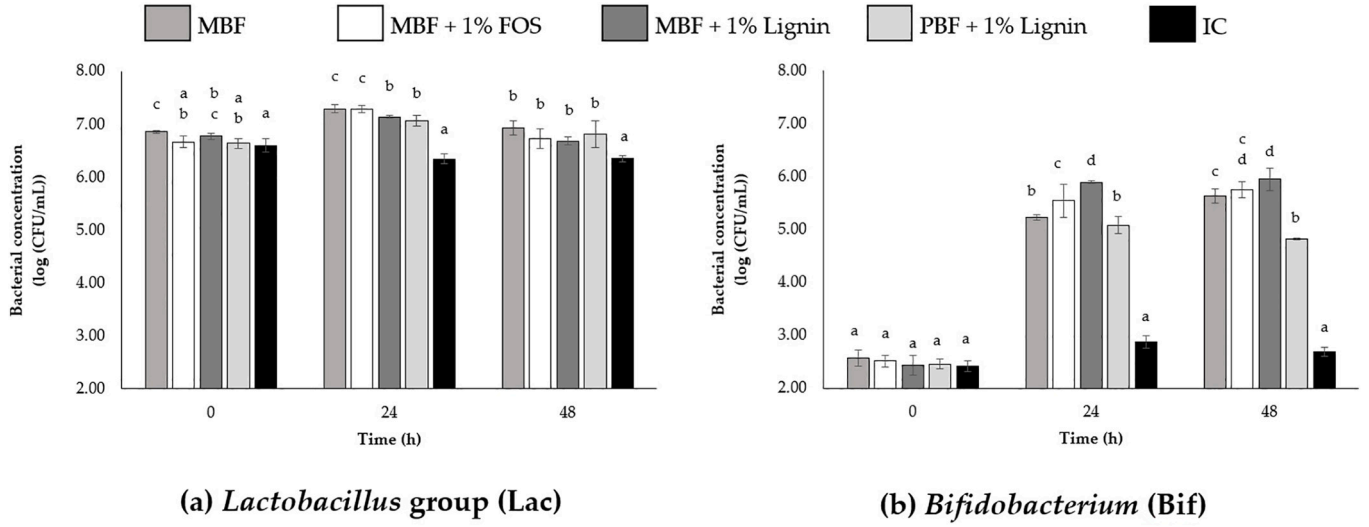


Fig. 5. Bacterial cell concentrations (log (CFU/mL), mean ± SD) of the *Lactobacillus* group (a) and *Bifidobacterium* (b) during the cecal fermentations for the different conditions tested. Different letters mark statically significant ($p < 0.05$) differences between conditions for each bacterial group at same sampling time. FOS – Fructo-oligosaccharides; IC – Inoculum control; MBF - Mash baseline feed; PBF - Pellet baseline feed.

Table 6
Short Chain Fatty acids concentrations (mM, means ± SD) produced during 48 h of cecal fermentation.

Condition	Time (h)	Concentration (mM)					
		Lactate	Acetate (A)	Propionate (P)	Butyrate (B)	Sum SCFA at 48 h (A + P + B)	Ratio A:P:B at 48 h
MBF	0	nd	nd	nd	nd	69	21:12:1
	24	49.98 ± 1.23^{b}	20.2 ± 0.2$^{a, b}$	9.0 ± 0.5$^{b, c}$	nd^{a}		
	48	nd^{a}	42.7 ± 0.8^{c}	24.4 ± 4.5$^{b, c}$	2.1 ± 0.7^{a}		
MBF + 1 % FOS	0	nd	nd	nd	nd	78	24:15:1
	24	54.1 ± 6.7^{a}	21.7 ± 1.6^{b}	8.6 ± 0.3^{b}	nd^{a}		
	48	nd^{a}	46.7 ± 1.0^{d}	29.4 ± 2.1^{c}	1.9 ± 0.1^{a}		
MBF + 1 % lignin	0	nd	nd	nd	nd	74	21:14:1
	24	44.4 ± 1.0^{b}	22.6 ± 2.2^{b}	10.0 ± 0.3^{c}	nd^{a}		
	48	nd^{a}	42.8 ± 1.4^{c}	29.5 ± 0.5^{c}	2.1 ± 0.5^{a}		
PBF + 1 % lignin	0	nd	nd	nd	nd	56	24:16:1
	24	68.6 ± 7.4^{c}	19.1 ± 1.6$^{a, b}$	6.0 ± 0.7^{a}	nd^{a}		
	48	40 ± 14.5^{b}	33.2 ± 0.1^{b}	21.6 ± 1.8^{b}	1.4 ± 0.4^{a}		
IC	0	nd	nd	nd	nd	32	6:2:1
	24	nd^{a}	17.9 ± 0.8^{a}	5.8 ± 0.3^{a}	3.8 ± 0.1^{b}		
	48	nd^{a}	21.6 ± 0.1^{a}	7.0 ± 0.3^{a}	3.4 ± 0.1^{b}		

Nd- not detected. Different letters mark statically significant ($p < 0.05$) differences between the different conditions at each sampling time. FOS – Fructo-oligosaccharides; IC – Inoculum control; MBF - Mash baseline feed; PBF - Pellet baseline feed.

3559.58 g, representing an increase of 3.2 %. Preferably, the pellets should have the ideal hardness to avoid being easily damaged but also should promote a suitable palatability and softness to improve livestock and poultry production performance [74]. Regarding the breakout peak, lignin induced a slight increase in the strength necessary to complete breakout of the pellets revealing a higher cohesion of the PBF + 1 % lignin compared to the PBF. As so, the force necessary for the breakout of the PBF was 4143.88 ± 1734.77 g and the PBF + 1 % lignin was 5786.15 ± 2411.14 g, representing an increase of 28.4 % (Fig. 4(b)). Lignin seems to act as a pellet binder, and as other type of similar ingredients reported in the literature, such as lignosulfonates, which are been used as dispersant in binder in animal feed, can promote an increase on pellets' hardness and consequently did not induced a negative effect on feed intake [75,76]. The resistance to pellets' deformation of the revealed that lignin incorporation slightly increased, namely 3.2 % (Table 5). This can be attributed to the ability of lignin as a binder to form new interparticle bonds that can be formed under pressure becomes higher, increasing the force and resistance to deformation. So far, lignin has never been investigated comprising its effect on the hardness of feed pellets, however being its natural function of support, it is expectable that its incorporation in some matrices, such as feed, can contribute to a higher hardness, however from the values herein obtained it is not significantly different from a standard pellet already used for chicken feed.

3.7. Prebiotic assessment

To evaluate the potential prebiotic of SCB lignin for poultry, an *in vitro* gastrointestinal model was carried out, simulating the avian gastrointestinal tract, and assessing the impact of the different chicken feeds under study on the growth of beneficial bacteria (i.e., *Lactobacillus* and *Bifidobacterium*), organic acids production and total ammonia production in the cecal fermentation.

The first step of prebiotic assay was to verify the impact of the incorporation of lignin in the mash chicken feed compared with the same mash chicken feed without supplementation and with a mash chicken feed supplemented with a well-known and recognized prebiotic in animal nutrition, FOS, that was used as positive control (condition 1 – MBF, condition 2 – MBF + 1 % FOS and condition 3 – MBF + 1 % lignin). The prebiotic potential of lignin was tested on different feed forms (mash - condition 3 - MBF +1 % lignin or pellet – condition 4 - PBF +1 % lignin). And it was assessed the impact on bacterial growth and behavior of the studied bacterial groups when there is no source of nutrients (condition 5 – IC).

3.7.1. Bacterial profile on the cecal fermentations

The Fig. 5 shows the bacterial populations of *Lactobacillus* and *Bifidobacterium* during cecum fermentations of the five mentioned conditions tested. In the cecal fermentations at 0 h, for all conditions, the concentration of *Lactobacillus* (6.6–6.9 log cell cyles) was higher than *Bifidobacterium* (2.4–2.6 log cell cycles). No significant differences ($p > 0.05$) were found at 0 h, between the five conditions tested for *Bifidobacterium*, however, for *Lactobacillus,* there were significant differences ($p > 0.05$) found between these conditions. The condition IC, in regard to the other conditions, is significantly different ($p < 0.05$) throughout the cecal fermentation, due to the decrease in the bacterial concentration of *Lactobacillus* and smaller growth of *Bifidobacterium*, being the condition with lowest concentration of these bacteria at time 24 (6.35 log cell cycles) and 2.87 log cell cycles, respectively) and 48 h (6.36 log cell cycles and 2.69 log cell cycles, respectively) (Fig. 5 and Appendix A - Table S2).

Regarding the impact on cecal *Lactobacillus*, it can be observed that at 24 h, the conditions that promoted the higher growth of these bacteria ($p < 0.05$) were the MBF (7.29 log cell cycles) and MBF + FOS (7.29 log cell cycles) conditions, while both conditions containing lignin had significantly ($p < 0.05$) lower growth (MBF + 1 % lignin, 7.14 log cell cycles and PBF + 1 % lignin, 7.07 log cell cycles in regard to these conditions. However, at 48 h, the conditions with feed had no significant differences ($p > 0.05$) between them, noting that the only statically significant difference ($p < 0.05$) observed is between feed and inoculum control.

Regarding the impact on cecal *Bifidobacterium*, at 24 h, the condition that promoted higher growth ($p < 0.05$) was MBF + 1 % lignin (5.89 log cell cycles), followed by MBF + 1 % FOS ((5.54 log cell cycles), MBF and PBF + 1 % lignin present similar values of growth and are not significantly different (5.22 log cell cycles and 5.08 log cell cycles, respectively). At 48 h, MBF + 1 % lignin was also the condition with the higher concentration of *Bifidobacterium* (5.95 log cell cycles), however it is not significantly different ($p > 0.05$) to the condition MBF + 1 % FOS (5.75 log cell cycles), followed by MBF (5.64 log cell cycles), which is not statistically different ($p > 0.05$) of MBF + 1 % FOS but it is statistically of PBF + 1 % lignin which presents the lower concentration ($p < 0.05$) of *Bifidobacterium* between the condition with feed (4.82 log cell cycles).

The results shown in Fig. 5 at time 0 h, for cecal *Lactobacillus* and *Bifidobacterium* are within the expected by the authors, since according to the scientific literature, *Lactobacillus* are a predominant bacterial genus of the avian cecal microbiota while *Bifidobacterium* is nonexistent in broiler cecum microbiota at early age, only appearing at 4 weeks of age, mostly due to environmental factors, dietary practices, and breed,

which can explain the reason of low amount of *Bifidobacterium* present in the cecal inoculum used in this experiment [77–80].

The IC condition, as would be expected, was the condition with the lowest bacterial growth since nutrients were not provided to promote the growth or maintenance of these bacteria throughout the fermentations. The impact of different feed conditions on *Lactobacillus* was most noticeable at 24 h, in which the condition with lignin promoted less growth compared to the other two conditions (MBF and MBF + FOS). However, at 48 h, all conditions that contain feed had the same promotion on the *Lactobacillus* growth. While for *Bifidobacterium*, MBF + 1 % lignin is the condition that most promoted bacterial growth, showing better results than FOS, which was used as a positive control, and having a greater potential when supplied in this form instead of a pellet form as in the PBF condition.

Lactobacillus and *Bifidobacterium* are saccharolytic bacteria that prefer simple short-chain sugars as carbon sources, thus competing between each other for the same type of nutrients [81–84]. According to several studies, prebiotics are safe feed additives to be incorporated in poultry diets without any adverse effect on their immune system, in which the dietary supplementation of FOS increases the proliferation of *Lactobacillus* and *Bifidobacterium* in chicken gut and even the supplementation of FOS at 0.5 % (w/w) increase the overall bacterial biodiversity, specially *Lachnospiraceae* and decrease of pathogenic bacteria, such as *Helicobacter* and *Desulfovibrio* [14,49,79,85]. Also a study of Baurhoo and co-workers [38], showed that the incorporation of 1.25 % (w/w) lignin to the broiler diets improved the cecal populations of *Lactobacillus* and *Bifidobacterium*. Thus, the results obtained in the present study confirm the potential of lignin and FOS to stimulate the growth of *Bifidobacterium* in the cecum of broilers.

These results indicate that the incorporation of lignin in poultry feed has the potential to promote *Bifidobacterium* growth in the broiler's cecum, when incorporated at 1 % (w/w) in a mash form (and not in a pellet form), thus being a prebiotic ingredient in poultry nutrition.

3.7.2. Cecal fermentation metabolic profile

The Table 6 shows the concentration of lactate, acetate, propionate, and butyrate produced throughout cecal fermentation carried out. The conditions which produced the highest levels of SCFA (i.e., acetate + propionate + butyrate) at the end of the experiment were MBF + 1 % FOS (78 mM) > MBF + 1 % lignin (74 mM) > MBF (69 mM) > PBF + 1 % lignin (56 mM) > IC (32 mM). The production pattern of the different SCFAs was identical between them (increase over time) while the lactate production pattern was different, with production and consumption throughout the fermentation (except for the IC condition, in which the production of lactate was not detected). At 24 h of fermentation, the production of SCFA is very similar between the different conditions tested (except for IC condition on butyrate production) and only at 48 h it is possible to visualize impacts on the production of these acids. For all the feed conditions tested, the concentration of lactate was higher at 24 h. The condition with higher concentration of lactate ($p < 0.05$) at 24 h was PBF + 1 % lignin (68.60 mM and 39.98 mM, respectively) while the results from the other feed conditions tested were not statistically different ($p > 0.05$) between each other. At 48 h, PBF + 1 % lignin was the only condition that was detected the presence of lactate (39.98 mM). Regarding the production of acetate, at 48 h, it is possible to observe that MBF + 1 % FOS is the condition with highest ($p < 0.05$) production (46.7 mM), followed by MBF + 1 % lignin (42.81 mM) and MBF (42.71 mM) which is not significantly different ($p > 0.05$) between each other, PBF + 1 % lignin (33.17 mM) and IC (21.56 mM). The conditions with the highest production ($p < 0.05$) of propionate, at 48 h, were the condition MBF + 1 % FOS (29.45 mM), MBF + 1 % lignin (29.47 mM) and MBF (24.44 mM), followed by PBF + 1 % lignin (21.60 mM) and IC (6.95 mM). Regarding the production of butyrate, IC condition is the only condition where butyrate was detected at 24 h (3.84 mM) and it was the condition with the highest ($p < 0.05$) value of butyrate at 48 h (3.44 mM), while the other conditions had similar and not significantly different ($p < 0.05$) values between each other at both sampling time points.

The type of substrate present during the cecal fermentations have a direct influence on cecal microbiota metabolism, which are dominantly saccharolytic and/or proteolytic bacteria [86,87]. The composition of the tested feeds is rich in carbohydrates and proteins; therefore, it is expected to be an increase in the bacterial metabolism. Among the metabolites produced during cecal fermentations of dietary fiber, carbohydrates, proteins and peptides, SCFA plays an important role for host's health, such as energy generation, mucin production, proliferation and growth of enterocyte, be a key player in the ability of *Bifidobacterium* inhibiting enteropathogens activity and intestinal immune response, among others [77–79,88]. Acetate, propionate and butyrate represent 90–95 % of the total SCFA produce in anaerobic fermentation [89]. Several mechanisms of action of prebiotics in the immunological resistance of the host GIT microbiota against pathogens have been discussed over the years, and one of the most mentioned is the promotion of bacterial growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*, which are bacteria that produce acetate and lactate and are indirectly related to the production of propionate (lactate is a substrate to the propionate production) and butyrate (acetate is a substrate to the butyrate production) [19,89,90].

Regarding the lactate values obtained in the conditions with feed, the condition that stands out the most is PBF + 1 % lignin, since it is the condition that has the highest lactate production and the only one that has, after 48 h of fermentation, a significant ($p < 0.05$) concentration of lactate. This result is unexpected since this is the feeding condition that least promotes the growth of the main lactate producers (*Lactobacillus* and *Bifidobacterium*). This means that the accumulation of lactate is being harmful to the growth of these bacteria. Lactate is a metabolite produced by several intestinal anaerobic bacteria from the fermentation of energy sources (especially carbohydrates), being a major fermentation product of *Lactobacillus* and *Bifidobacterium*, that produce this metabolite to achieve overall redox balance [91]. Lactate is an organic acid usually produced in intestinal anaerobic fermentation in the first hours of fermentation, disappearing over the course of fermentation due to its consumption by gut microorganisms (e.g., *Bacteroides* and *Roseburia*), which converts lactate into acetate, propionate, and butyrate. Normally, lactate does not accumulate in healthy gut lumen [91]. This metabolic process is known as cross-feeding and limits the accumulation of lactate, preventing metabolic acidosis which is an important issue in animal nutrition [89,91,92]. Lactate-utilizing bacteria plays a major role in maintain intestinal health by preventing accumulation of lactate and subsequent microbial perturbations in the cecal microbiota, since lactate exert both beneficial and harmful effects on the gut environment [91]. The cecal microbiota bacteria are important for disease prevention due to its role of maintaining the balance between lactate production and consumption in the host's gut. A cross-feeding phenomenon of lactate was observed in all conditions except for IC in which the lactate was not detected during the fermentation.

Regarding SCFA results, lignin incorporated in mash feed has a similar SCFA production to the mash feed incorporated with FOS, even though mash feed supplemented with FOS produces a higher ($p < 0.05$) value of acetate. The feed form that lignin is incorporated has an impact on the production of SCFA, and the mash feed with lignin has higher values than the values of the pelleted form. These results are consistent with the results of bacterial growth performed in this study, since the conditions that promoted higher growth of *Bifidobacterium* were MBF + 1 % lignin and MBF + 1 % FOS, since production of SCFA promote the proliferation of beneficial bacteria (e.g., *Lactobacillus* and *Bifidobacterium*) in poultry cecal microbiota [20]. It is also in agreement with the results obtained from lactate production, in which these conditions did not have lactate accumulation while condition PBF + 1 % lignin has lactate accumulation and is the condition with the lowest SCFA production of the tested feed conditions, since lactate is used as a substrate to produce SCFA.

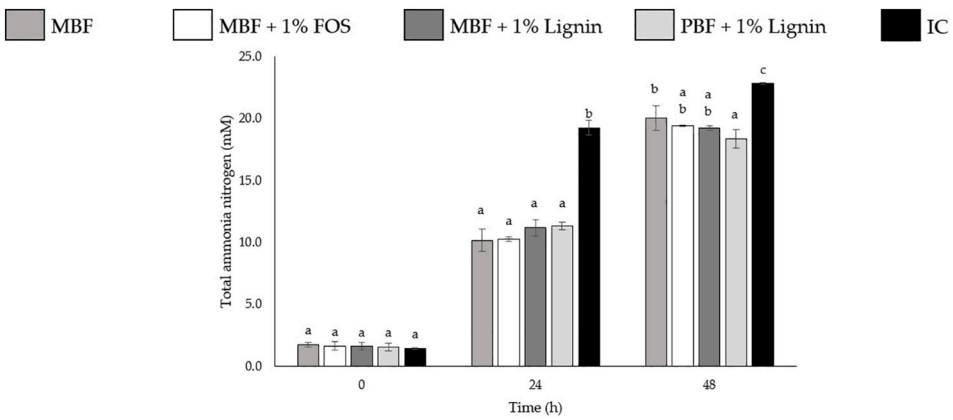


Fig. 6. Concentration of total ammonia nitrogen (mM, means ± SD) produced during 48 h of cecal fermentation. Different letters mark significant ($p < 0.05$) differences between each condition at same sampling time. FOS – Fructo-oligosaccharides; IC – Inoculum control; MBF - Mash baseline feed; PBF - Pellet baseline feed.

In this study, the incorporation of FOS and lignin at 1 % (w/w) in mash feed, did not promote the production of broiler cecal SCFA while pellet form feed supplemented with lignin at 1 % (w/w) had a lower production of cecal SCFA in comparison with mash feed supplemented with lignin, indicating that mash feed promote more cecal SCFA that pellet feed.

3.7.3. Assessment of the total ammonia nitrogen profile

In Fig. 6 shows the concentration of total ammonia nitrogen produced throughout the cecal fermentations. At 0 h of fermentation, the total ammonia nitrogen values are the same ($p > 0.05$) on all conditions and begin to differ ($p < 0.05$) at 24 h between the IC condition and the other four feed conditions tested. This trend continues to maintain at 48 h, with the IC condition being the condition with the highest ($p < 0.05$) production of total ammonia nitrogen.

Ammonia is a metabolite produced by the proteolytic activity of the intestinal microbiota, used as nitrogen source by the microbiota and it is mostly excreted via the stool and urine or absorbed in the gut. However, depending on its concentration in the gut, it can be toxic to the host [93,94]. Fermentation and production of SCFA, thru the reduction of pH, decreased the formation of ammonia driven from the amino acids deamination [92,94,95]. This is consistent with the results of the SCFA measurement since the condition with the lowest SCFA production was IC and this condition is the one with more total ammonia nitrogen produced while the other conditions had similar SCFA values produced, and recorded values similar values of total ammonia nitrogen produced. However, PBF + 1% lignin had the lower value ($p < 0.05$) of total ammonia nitrogen produced in all conditions and had lower SCFA production between the feed conditions tested which would not be expected.

The results show in Fig. 6 indicate that lignin, regardless of the form it is incorporated into the poultry feed, does not increase intestinal ammonia production. This is relevant since ammonia is one of the main gas produced in poultry houses and it is the greatest environmental concern for the poultry industry, due to negative impact ammonia have on the health and welfare of animals (e.g., discomfort and increase of susceptibility for diseases) and poultry house workers (e.g., damage on the respiratory system) and also to the environment [96–98].

4. Conclusions

Sugarcane bagasse lignin revealed not affect significantly several physico-chemical properties of both chicken feed forms, mash and pellets. The main differences were found in the capacity of SCB lignin to decrease the tendency of the pellets for microbial contamination and to increase its tensile strength, resistance to deformation and disintegration time. In fact, SCB lignin revealed to act as a binder, being responsible for the differences founded in pellet's mechanical properties and disintegration.

Results revealed that SCB lignin presents potential as animal feed additive when supplemented in mash feed diets. It promotes *Bifidobacterium* growth, a well-known beneficial bacteria inhabiting the gut microbiota, whose activity, among others, inhibits the growth of pathogenic bacteria (e.g., *Enterobacteriaceae*), an important factor for the livestock industry. At the same time, data showed that, without the need of a profound change in animals' diet, this supplementation can deliver results, at the gut microbiota level, that are in agreement with the animal's well-being and health maintenance. Therefore, this study suggests SCB lignin as a promising sustainable and eco-friendly feed additive to chicken feed.

CRediT authorship contribution statement

Joana F. Fangueiro: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing–original draft. Nelson Mota de Carvalho: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing–original draft. Filipa Antunes: Investigation, Methodology. Inês F. Mota: Investigation, Methodology. Manuela Estevez Pintado: Funding acquisition, Project administration, Resources. Ana Raquel Madureira: Funding acquisition, Project administration, Resources, Supervision, Validation, Writing–review & editing. Patrícia Santos Costa: Funding acquisition, Project administration, Resources, Supervision, Validation, Writing–review & editing. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

Patricia Costa has patent LIGNIN, METHODS OF EXTRACTION AND USES THEREOF pending to UNIVERSIDADE CATÓLICA PORTUGUESA - UCP, AMYRIS BIO PRODUCTS PORTUGAL UNIPESSOAL LDA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.124262.

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