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Comparative analysis of pre-treatment strategies and bacterial strain efficiency for improvement of feather hydrolysis

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Abstract

Background Feathers are a major by-product of the poultry industry, which poses an environmental challenge due to the recalcitrant structure of keratin, making them resistant to degradation. Traditional methods of feather handling, like conversion to feather meal, are energy-intensive and have limited efficiency. Biotechnological approaches, particularly microbial hydrolysis, offer a novel and more sustainable alternative for keratin degradation. This study evaluated feather hydrolysis by two bacterial strains, newly characterized cold-adapted *Arthrobacter oryzae* (BIM B-1663) and *Bacillus licheniformis* (CCM 2145^T), known as a keratin degrader, under various feather pre-treatment conditions, including washing, autoclaving, drying, and grinding.

Results Both bacterial strains were able to degrade pretreated feathers with a degradation efficiency of 75 to 90%, resulting in high ratios of nitrogen to carbon in the hydrolysates. *B. licheniformis* confirmed its enzymatic capabilities with high levels of general and specific protease activity and furthermore presented enriched amounts of amino acids of industrial interest. *A. oryzae* showed a much higher keratinase/protease activity ratio, demonstrating high specificity and efficiency of its enzymes. Autoclaving emerged as the most important determinant of microbial degradation efficiency and influenced the composition (peptide pattern, amino acid content, and chemical composition assessed through FTIR) of the resulting hydrolysates. Feather drying, although not improving microbial degradation efficiencies, had a considerable impact on hydrolysate composition.

Conclusions The results show that both tested bacterial strains can efficiently degrade autoclaved feathers but use distinct enzymatic strategies to do so. Enriched profiles in amino acids and high nitrogen content in the hydrolysates also advocate for the benefits of microbial feather hydrolysis over an enzymatic one. To the authors' knowledge this study is the first to report a comprehensive evaluation of the impact of various feather pre-treatment methods on the efficiency of subsequent microbial feather hydrolysis and is the first one to report enrichment in phenylalanine, lysine, and tyrosine secreted by *B. licheniformis*.

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Background

Feathers, accounting for about 8 billion tons of by-products generated annually worldwide [15], pose a significant environmental and sustainability challenge due to the difficulty of processing this material. Even though some parts of it are converted into nitrogen rich hydrolysates for fermentation [5, 37] and some commercial hydrolysates are already available on the market, such as those produced by Bioextrax AB (Lund, Sweden), the most common fate of feather materials is incineration and landfill disposal. Only small portions of feather materials are converted into feather meal for animal feed [7]. Feather meal conversion is a costly process due to the need for extensive treatment involving washing, autoclaving, drying, and grinding, all of which are energy-intensive [33]. Moreover, feather meal is considered to have low nutritional value due to the low accessibility of nitrogen from keratin, which constitutes more than 90% of the total composition of feathers [4]. Keratin is a recalcitrant protein with a pleated secondary structure and intermolecular disulfide bonds, which make it highly resistant to decomposition [14, 32].

Hydrolysis of the feathers could potentially increase their value and can be performed using physical and chemical methods, such as alkali or acid treatments, and steam explosion. However, these techniques often suffer from limitations in efficiency and specificity, and have a negative environmental impact [7, 32]. Biotechnological approaches using crude enzymes [20, 27, 37] or microorganisms that produce keratin-degrading enzymes offer a more sustainable solution [19]. Microbial keratinases, a specific group of proteolytic enzymes that convert keratin into shorter peptides and amino acids, are the only proteases capable of effectively hydrolyzing keratin wastes [24]. The keratin-degrading potential of bacteria and fungi has been well-documented [19], and dermatophyte filamentous fungi are considered to be particularly efficient.

As for bacterial keratinolysis, studies on *Bacillus* have shown that species such as *Bacillus licheniformis* secrete efficient keratinases [47]. The keratinolytic efficiency of *B. licheniformis* has been widely studied [3, 44], and the highest keratinolytic performance has been observed at 35 °C. Its purified enzymes have proven to be valuable in biotechnological applications and this strain has been extensively utilized for keratinase production [34, 45]. Recently, it has been suggested that cold-adapted bacteria, such as *Arthrobacter oryzae*, enable more energy- and cost-efficient keratin hydrolysis. *A. oryzae* is a cold-adapted strain isolated from green snow in Eastern Antarctica, has demonstrated efficient keratin degradation at temperatures below 30 °C [38].

The effects of various pre-treatment techniques on feather enzymatic hydrolysis of feathers have been investigated in several studies, including methods like microwave-alkali treatment [23] and thermal alkaline pre-treatment [10]. So far, studies on microbial hydrolysis have typically been using feather meal as the starting material rather than raw feathers. The production of feather meal from raw feathers involves four pre-treatment steps (washing, autoclaving, drying, and grinding), each of which alters the structural integrity of the keratin in feathers. These modifications may affect the efficiency of microbial hydrolysis and, consequently, the composition of resulting hydrolysates. Moreover, these pre-treatments are time-consuming and energy-intensive and pose challenges in scaling up the process to an industrial level. Additionally, different microbial strains may respond differently to feather material processed using specific pre-treatment conditions, influencing their keratinolytic activity [22]. A thorough understanding of the impact of pre-treatment procedures on microbial feather hydrolysis can help to better optimize and increase the efficiency of the process and can help to reduce the need for all these pre-treatment steps and may yield processes with increased efficiency that are eco-friendlier and more cost-effective. To the authors' knowledge, this study is the first to report a comprehensive evaluation of the impact of feather pre-treatment methods (washing, autoclaving, drying, and grinding) on the efficiency of microbial feather hydrolysis.

In this study, two bacterial strains, *Arthrobacter oryzae* (BIM B-1663) and *Bacillus licheniformis* (CCM 2145^T), were used for microbial feather hydrolysis. *Arthrobacter oryzae* (BIM B-1663) is a strain newly isolated from Antarctic green snow that has recently been thoroughly characterized [1, 2, 38–40]. This strain was compared with the benchmark strain *Bacillus licheniformis* (CCM 2145^T). The efficiency of microbial hydrolysis was evaluated based on the degree of feather degradation, proteolytic activity in culture media, and the composition of the obtained hydrolysates. Degradation efficiency was assessed by measuring the remaining non-degraded feather material, and hydrolysates were analyzed using size exclusion chromatography (SEC). Enzymatic activity was measured in terms of total protease and keratinase activity. Finally, a compositional analysis of the hydrolysates was performed that included elemental analysis and analysis of the amino acid profiles, while Fourier transform infrared spectroscopy (FTIR) was used to investigate the total biochemical composition of the hydrolysates.

Methods

Bacterial strains

Two strains, *Arthrobacter oryzae* BIM B-1663 from the Belarussian Collection of Non-pathogenic Microorganisms (Institute of Microbiology of the National Academy

of Science of Belarus) and *Bacillus licheniformis* CCM 2145^T from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), were used in the study. The bacteria stock cultures were prepared by growing bacteria overnight at 25 °C, 150 rpm, in Brain Heart Infusion (BHI) (Sigma-Aldrich, USA, Cat. No. 53286). 750 µL of the bacterial cell suspension was used in a 20% (v/v) glycerol, and stored at – 80 °C.

Pre-treatment procedures of feather material

The raw feather material was provided by Norilia AS (Oslo, Norway). In the present study, we tested the following feather pre-treatment methods (Table 1): (1) Washing (W), which was done for raw feathers using warm running tap water until feathers were visually clean, no detergents were used; (2) Autoclaving (A), which was done for washed or raw un-washed feathers at 130 °C, 3 atm for 40 min; (3) Drying (D), which was done by incubation at 100 °C for 5 h and (4) Grinding (G) which was performed using a kitchen multi-blender CW1949 (Coline, China), followed by sieving, using a sieve shaker Retsch AS 200 (Retsch GmbH, Verder scientific, Germany) equipped with stainless steel sieves with mesh sieve size 500 and 250 µm to obtain feather meal with a particle size of less than 250 µm. The pre-treatment methods were used alone for washing and autoclaving, and in a sequence, as described in Table 1. For experiments in which autoclaving was the only pre-treatment step, raw feathers were autoclaved directly together with other medium components. In the case of dried feathers and feather meal, the feather material underwent an initial autoclaving step before drying and/or grinding, followed by a second autoclaving step together with other medium components.

Bacterial hydrolysis of feather material

Bacterial inoculums were prepared by, first, recovering bacteria from the stock cultures, using brain heart infusion (BHI) agar (Sigma-Aldrich, USA) and incubating for seven days at 35 °C–25 °C for *B. licheniformis* and *A. oryzae*, respectively. Then, a single colony was transferred into 100 mL of BHI broth (Sigma-Aldrich, USA) and incubated in a MaxQ 4000 orbital shaker incubator (Thermo Fisher Scientific, USA) with 150 rpm agitation (1.9 cm circular orbit) for 24 h at 25 °C for *A. oryzae* and 35 °C for *B. licheniformis*.

The composition of the medium containing feather material was prepared according to Smirnova et al., [38] (in g/L): $MgSO_4 \times 7H_2O$ – 2; KH_2PO_4–0.1; $FeSO_4 \times 7H_2O$ – 0.01; $CaCl_2 \times 2H_2O$ – 0.13; yeast extract – 0.5. The amount of feather material in each flask was equivalent to 10 g/L dry weight for the pre-treatment methods WAD and WADG. For media with feather material pretreated according to A, W, and WA, 13.06 g/L of feather material was used, which corresponds to 10 g/L of dried feathers. It was measured that during drying the weight of the feather material decreases by 23.4%, hence 13.06 g/L. For the media of A, WA, WAD, and WADG, feather material was autoclaved together with the other media components at 121 °C, 1.023 atm for 20 min. For the medium with feathers treated by solely washing (W), feather material was added directly to the autoclaved medium components.

Erlenmeyer flasks of 250 mL were filled with 75 mL of the different feather media, inoculated with 2.6 mL of bacterial BHI inoculum, and incubated with 150 rpm agitation (1.9 cm circular orbit), at 25 °C and 7 days for *A. oryzae*, and at 35 °C and 5 days for *B. licheniformis*. Temperature and incubation time were selected according to the results obtained in the previous study [38]. All hydrolysis experiments were performed in three biologically independent replicates.

Assessment of feather degradation efficiency and production of bacterial mass

To assess feather degradation efficiency for media with differently pretreated feather material, cultures after cultivation in Erlenmeyer shake flasks were transferred into sterile 50 mL falcon tubes and centrifuged at 4696 g for 10 min at 4 °C to separate hydrolysate from non-degraded feather material and bacterial biomass. The obtained pellet underwent filtration through pre-weighted cellulose nitrate filters with, first, 5 µm pore size (VWR, USA) to separate non-degraded feather material and then, with

Table 1 List of samples and sample codes according to the pre-treatment methods (W-washing, A- autoclaving, D- drying, and G-grinding) applied to feather material. Cells with X represents the application of pre-treatment type to feathers

Sample code	Washing	Autoclaving	Drying	Grinding
W	X			
A		X		
WA	X	X		
WAD	X	X	X	
WADG	X	X	X	X

0.8 µm pore size filter (VWR, USA) to separate bacterial biomass. Filters were dried overnight at 95 °C and weighed before and after filtration to subtract the filter weight from the final non-degraded feather meal and cell mass. The feather degradation efficiency was estimated by calculating the percentage of the non-degraded feather material over the total initial load of feather material.

Non-specific proteolytic activity

The protease solutions used for standard calibration (protease from bovine pancreas and proteinase K) were assessed using Sigma's non-specific protease activity assay using casein as a substrate [11].

The proteolytic activity in the hydrolysate samples was measured using a 96-well plate assay using azocasein (Sigma-Aldrich, USA) as a substrate following Caldas et al. [6, 38] using protease from bovine pancreas (Cat. No P4630) as a standard. All samples were analyzed in triplicates, with absorbance at 440 nm measured using a SPECTROstar Nano spectrophotometer (BMG Labtech, Germany). A blank with distilled water was processed in parallel, to account for background absorbance.

Keratinase activity

To assess keratinase activity, azo-keratin was employed as a substrate. Azo-keratin was prepared from feathers (mainly β-keratin) similarly as in Smirnova et al., [38] following Herzog et al., [17] Due to the low signal detection, the samples of *A. oryzae* cultures were concentrated 3-fold using Amicon® Ultra centrifugal 10 kDa MWCO filters (Sigma-Aldrich, USA). The keratinase activity assay was performed in the following way: 10 mg of azo-keratin powder was mixed with 0.9 mL of 50 mM potassium phosphate buffer pH 7.5 and 0.1 mL of sample. The reaction mixture was incubated for 1 h at 37 °C and 800 rpm, consuming a fraction of the substrate in all reactions. After incubation, the reaction mixture was centrifuged at 11.510 g for 5 min at 4 °C. Finally, 0.9 mL of the supernatant was transferred into a semi-macro cuvette (1 mL, 1 cm path length), and the absorbance was measured at 415 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech, Germany). A blank was prepared by replacing the volume of the sample by distilled water in the reaction mixture and underwent the same processing steps to account for any background absorbance. Proteinase K (Sigma-Aldrich, USA, Cat. No. P5380) was used for standard calibration. All samples were analyzed in two technical replicates.

Elemental and total amino acid analysis

The bacterial biomass from overnight BHI cultures was washed by three cycles of centrifugation, supernatant discard, and resuspension in distilled water. These washed bacterial biomasses were used only through elemental amino acid analysis. The feather meal medium (CM), containing all the salts and feather meal, feather meal (FM), and hydrolysates were used for amino acid analysis and elemental analysis. Prior to these analyses, the samples were dried using freeze-drying in a Labconco FreeZone2 2.5 L (LabConco, USA) at -53 °C 0.052 mBar.

For the total amino acid analysis, the samples underwent an oxidation step before the acid hydrolysis, required for cysteine and cystine determination. Tryptophan was not determined due to oxidative degradation. Amino acids were quantified using a Biochrom 30 + Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK). This procedure was performed following the analytical method outlined in Commission Regulation (EC) No 152/2009 (2009).

The total carbon, hydrogen, nitrogen, and sulfur content were estimated using the Dumas method, following ISO 16,634 (2008). The instrument used was a Vario El Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The determination of macro and micro elements (Macro: Ca, K, Mg, Na, P, and Micro: Fe, Mn, Cu, and Zn) was carried out first by digesting dried hydrolysates followed by a spectrophotometric analysis with an MP-AES (Microwave Plasma Atomic Emission Spectrometer) from Agilent in accordance with the analytical methods outlined in Commission Regulation (EC) No 152/2009.

Determination of free sulfites and thiol groups

Free sulfites and thiol groups in the hydrolysates were quantified using the Total and free sulfite assay kit (Neogen, USA, Cat # K-SULPH). Free sulfite levels were assayed by Schiff reagent and total sulfites by Ellman's reagent. The assays were conducted in technical triplicates on 96-well plates, following the manufacturer's instructions, except for the use of 105 µL of undiluted samples instead of diluted samples. Fresh standards of $NaSO_3$, diluted in 0.1% (w/w) citric acid, were prepared for standard calibration. Thiol content was calculated by subtracting the free sulfite value from the total sulfite values.

Size exclusion chromatography

Size-exclusion chromatography (SEC) was employed to assess the molecular weight distribution of peptides present in the hydrolysates. The hydrolysate samples were first freeze-dried as described above. The samples were then dissolved in distilled water to reach a concentration of 10 mg/mL of dry hydrolysate and filtered using 0.45 µm PVDF syringe filter, making them ready for injection. Chromatographic separation was carried out using a Dionex UltiMate 3000 Standard System (Thermo Fisher Scientific, Waltham, MA, USA). Twelve compounds of different sizes were used as standards

(Bovine serum albumin, albumin from chicken egg white, carbonic anhydrase from bovine erythrocytes, lysozyme, cytochrome-C from bovine heart, aprotinin from bovine lung, insulin chain-B oxidized from bovine pancreas, angiotensin II human, bradykinin fragment 1–7, [DAla2]-leucine encephalin, Val-Tyr-Val and tryptophan). Both standards and hydrolysate samples (analyzed in two replicates) were separated at 25 °C on a BioSep-SEC-s2000 column (Phenomenex, 300 × 7.8 mm) with an injection volume of 10 µL for the standards and 20 µL for the hydrolysates. The mobile phase was composed of acetonitrile and ultrapure water in a 30:70 (v/v) ratio, containing 0.05% trifluoroacetic acid (TFA). For isocratic elution, a flow rate of 0.9 mL/min was maintained for 20 min. At 20.1 min, the mobile phase was switched to NaH_2PO_4 (0.10 M) for column cleaning, which continued until 23 min. Between 23.0 and 23.1 min, the initial elution conditions were restored, followed by column re-equilibration for an additional 42 min. Chromatographic runs were controlled using Chromeleon™ Chromatography Data System software (Thermo Fisher Scientific, Waltham, MA, USA). For chromatographic runs of both the standards and hydrolysates, a UV trace of 214 nm was recorded. SEC chromatograms of the hydrolysates were converted to molecular weight distributions, and weight average molecular weights (Mw) were calculated using a calibration curve constructed using molecular weight standards. These calculations were performed in MATLAB (R2022b, The Mathworks Inc.) using the openly available SEC2MWD toolbox [26]. All samples were analyzed in triplicate, and the average values were reported.

Fourier transform infrared spectroscopy (FTIR) analysis

The hydrolysate samples were subjected to FTIR spectroscopy analysis, where 10 µL of a sample was deposited on a 384-well silicon microplate. After drying, the deposition of 10 µL and subsequent drying was repeated twice to reach a total volume of 30 µL per sample. FTIR spectroscopy measurements were carried out in transmission mode using a high-throughput screening extension unit (HTS-XT) coupled to a Vertex 70 FTIR spectrometer (both Bruker Optik, Germany). The FTIR system is equipped with a global mid-IR source and a deuterated L-alanine doped triglycine sulfate (DLaTGS) detector. The spectra were recorded with a total of 64 scans, utilizing Blackman-Harris 3-Term apodization, a spectral resolution of 6 cm^{-1}, and a digital spacing of 1.928 cm^{-1}, covering the range from 4000 to 400 cm^{-1}, and employing an aperture of 6 mm. Spectra were recorded as the ratio of the sample spectrum to the background spectrum of the empty IR transparent microplate. Each sample was measured in three technical replicates. Data acquisition and instrument control were facilitated using OPUS 8.2 software (Bruker Optik GmbH, Germany).

Data analysis

FTIR data were analyzed using the Orange data mining toolbox version 3.32.0 (University of Ljubjana, Slovenia) [12]. Data analysis of FTIR spectra was done in the following way: (1) preprocessing with extended multiplicative signal correction (EMSC) with linear and quadratic terms [21, 43], and (2) principal component analysis (PCA) using the whole spectral region 4000 to 400 cm^{-1} of the preprocessed spectra.

Scanning electron microscopy

In order to visualize surface-associated changes in feathers after applying different pre-treatment methods, feathers were first sputter coated with 50 nm of platinum using a Leica EM ACE200 to ensure conduction. Scanning electron microscopy (SEM) imaging was conducted with a Zeiss EVO 50 scanning electron microscope (Carl Zeiss, Cambridge, UK) at an accelerating voltage of 5 kV in secondary emission mode.

Results and discussion

Impact of pre-treatment on feather surface structure

Scanning electron microscopy (SEM) analysis revealed the effects of the tested pre-treatments on the overall surface structure of feathers (Fig. 1). The comparison between raw and washed feathers indicated that the washing process significantly reduces the number of particles (e.g. blood, lipids, dust) adhering to the feather's surface, resulting in a cleaner appearance (Fig. 1A and B). This washing step not only removes some of the surface particles but also separates the barbules of the feather, thus changing the feather structure slightly. The pressure and high temperature applied during autoclaving after washing (Fig. 1C) resulted in noticeable fusion and smoothing of the barbules. Feathers that underwent washing, autoclaving, and drying exhibited the most modified surface texture (Fig. 1D). This combination of treatments significantly alters the feather's structural characteristics, leading to a smoother and more exposed surface. These changes are also noticeable in the feather's texture, with autoclaving making feathers more flexible and drying leading to a more friable feather material.

However, these pre-treatments alter the structure of feathers less than some other existing treatments, such as chemical alkali, resulting in shredded feather material with considerably more broken structure [9].

Autoclaving creates a smooth, exposed feather surface, which may influence subsequent microbial hydrolysis. While an increased surface area enhances enzymatic accessibility, it may also promote bacterial adhesion. Previous studies suggest that *Bacillus licheniformis* benefits from cell adhesion during feather degradation [36]. Furthermore, the bio-membrane hypothesis [32] proposes some particular degradation mechanisms occurring in

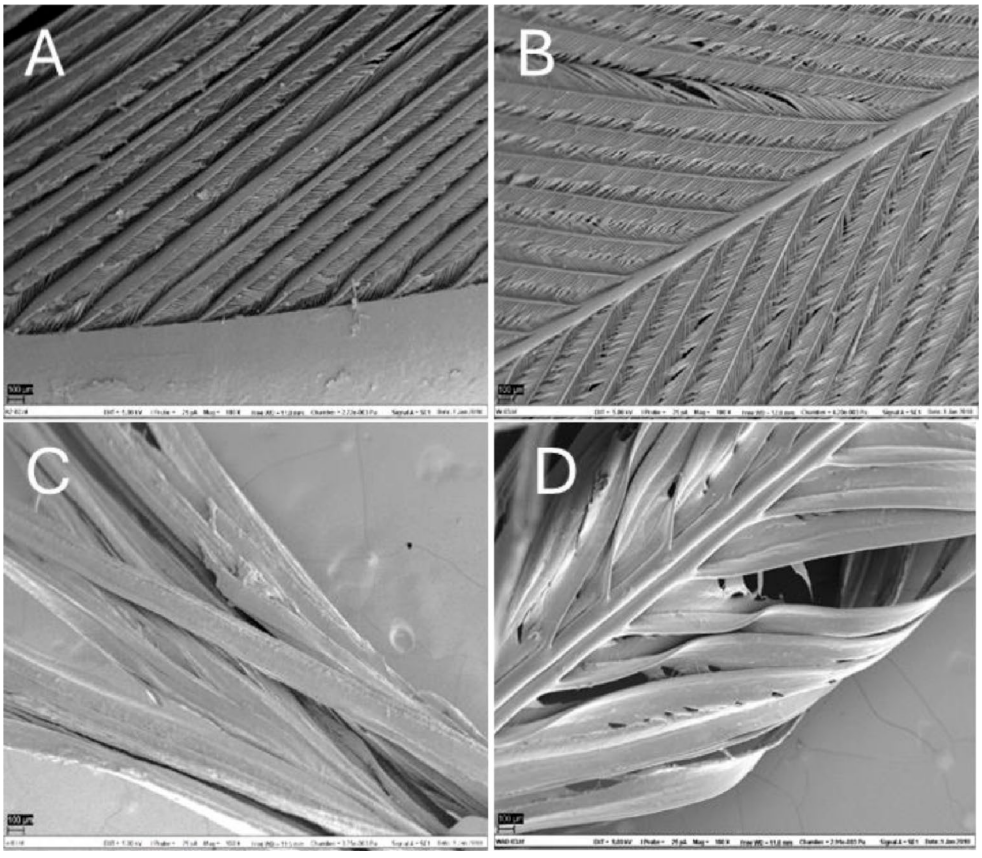

Fig. 1 SEM images of raw (**A**), washed (**B**), washed, and autoclaved (**C**) feather, and washed, autoclaved, and dried feather (**D**). Magnification of 100X

direct contact with keratinous material where cell-bound redox systems facilitate disulfide bond cleavage.

Feather degradation efficiency

The efficiency of feather microbial degradation after using various pre-treatment methods was assessed by calculating the percentage of degraded feather material after microbial hydrolysis. Both bacterial strains exhibited the lowest degradation efficiency (approximately 75%) with the raw washed feathers (W) (Fig. 2A). This suggests that the presence of leftover surface contaminants that are not removed by washing, or the unaltered structural integrity of the raw feathers may inhibit bacterial access to keratin.

In contrast, Fig. 2A shows that both bacteria demonstrated efficient feather degradation when using feathers that went through at least a step of autoclaving (A, WA, WAD, and WADG), with *B. licheniformis* generally achieving slightly higher rates (maximally reaching 91% for WA and A) than *A. oryzae*, (maximally reaching 85% for A). Autoclaving, both alone (A) and in combination with washing (WA), resulted in the highest degree of feather degradation for both bacterial strains (83–91%, Fig. 2A). This increase in degradation efficiency can likely be attributed to the autoclaving process, which effectively disrupts the feather's structural integrity, as seen in the SEM images (Fig. 1), likely making keratin more accessible for bacterial degradation. Furthermore, the fact that *B. licheniformis* performed particularly well when feather material was autoclaved and washed may be attributed to the synergy between the two steps. Indeed, autoclaving might lead to a physical breakdown of the barbules and keratin denaturation while washing removed contaminants, enabling the separation of barbules. This likely facilitated better substrate accessibility.

When feather material underwent additional treatments in addition to autoclaving, such as drying and grinding (WADG) or only drying (WAD), the degradation efficiency for *B. licheniformis* slightly decreased from 91 to 84% (for both WAD and WADG) (Fig. 2A). For *A. oryzae*, the second least effective feather pre-treatment was washed, autoclaved, and dried feather (WAD) whereas the hydrolysates using feather meal (WADG) showed higher degradation with 81% (Fig. 2A). However, the degradation efficiency calculated from non-degraded keratin particles is limited to the weight of particle of more than 5 µm and is to be put in perspective with the dry weights of the hydrolysates (Additional files 1, Table S1). Indeed, the dried mass after hydrolyzation is increased for both bacteria after autoclaving but also

Fig. 2 **A** – Feather degradation efficiency for microbial hydrolysis of feather materials. The feathers were pretreated by autoclaving (A), washing (W), washing and autoclaving (WA), washing, autoclaving and drying (WAD), and washing, autoclaving, drying, and grinding (WADG). The results are shown for 5 days of growth of *B. licheniformis* (green) and 7 days of growth of *A. oryzae* (purple). **B** – Bacterial biomass (cell dry weight (CDW)) obtained after 5 and 7 days of bacterial hydrolysis, respectively, for *B. licheniformis* and *A. oryzae*. The standard deviation was calculated between the biological triplicates of the produced hydrolysates

after drying, indicating that more particles are in solution. This can indicate that the drying process leads to changes in the feathers' surface and/or the structure and, thus, hydrolysis of keratin. Taken together, the results obtained with both bacteria show that autoclaving not only sanitizes the material for subsequent use in fermentation but is also the key step needed to improve feather hydrolysis.

The cell dry weight of produced bacterial biomass in the hydrolysates was also assessed, revealing differences between the two bacterial strains (Fig. 2B). *A. oryzae* consistently exhibited higher cell mass compared to *B. licheniformis* under all tested conditions, except for the WAD condition (with 0.28 to 0.39 g/L of biomass for *B. licheniformis* and 0.33 to 0.59 g/L of biomass for *A. oryzae*). These differences were already highlighted in the previous study [38], where results comparing the bacterial growth and degradation efficiency of feather meal showed that bacterial biomass levels were reaching their maxima after 2 days of cultivation, with the highest values for *B. licheniformis*. These maxima of cell dry weight were followed by a sharp decline of the cell biomass for *B. licheniformis* and a slower one for *A. oryzae*, resulting in *B. licheniformis* having the lowest cell dry weights in the hydrolysates at 5 and 7 days.

To the authors' knowledge, this study is the first to report on the impact of the different feather pre-treatments involved in the process of turning raw intact feathers into feather meal and their combinations on bacterial hydrolysis. Previous studies have reported on the impact of pre-treatments, such as alkaline treatment, on enzymatic hydrolysis of feather [10] as well as on the effect of microwave-water, autoclave-alkali, and heating-alkali treatment on feathers structure [23].

Non-specific protease activity

Measurement of total protease activity via the azocasein assay revealed significant differences between the two bacterial strains and across the five tested feather pre-treatment conditions (Fig. 3). *B. licheniformis* demonstrated proteases activity ranging from 806 to 981 mU/mL, except in samples obtained from the hydrolysis of non-autoclaved feathers (W) where the protease activity was measured at 51.84 mU/mL. The highest activity for *B. licheniformis* was observed in the cultures with WADG treated feathers, while the lowest activity of 51.84 mU/mL occurred in the samples from the hydrolysis of

Fig. 3 Non-specific proteolytic activity in culture supernatant of hydrolysate samples. The feathers were pretreated by autoclaving (A), washing (W), washing and autoclaving (WA), washing, autoclaving and drying (WAD), and washing, autoclaving, drying and grinding (WADG). The results are shown for **A**: 7 days growth of *A. oryzae* and **B**: 5 days growth of *B. licheniformis*. The standard deviation was calculated between the biological and technical triplicates. Note the scale difference in the y axis

washed feathers (W). *A. oryzae*, displayed protease activity averaging around 44.5 mU/mL for all feather pretreatments, except for the non-autoclaved feathers. The highest activity for *A. oryzae* was found in the culture with WAD feathers (46.51 mU/mL), and the lowest activity (35.22 mU/mL) was recorded for the samples for the culture using washed feathers (W). The results reported for the cultures with feather meal (WADG) show slightly higher activity than the ones reported by Smirnova et al., [38] When comparing the two strains, *B. licheniformis* consistently exhibited higher protease activity than *A. oryzae* under all conditions. Additionally, the protease activity of *A. oryzae* showed less variation across the tested feather pre-treatment conditions. Notably, feathers pretreated with WADG resulted in the highest protease activity for both bacteria, with a more pronounced effect seen for *B. licheniformis*.

The low total protease activity observed for washed feathers (W) for both bacteria suggests that simple washing of feathers is insufficient for inducing optimal enzymatic activity of bacteria (Fig. 3). This condition leaves keratin in its native, highly cross-linked state, making it less accessible for protease degradation, which could also mean that the level of released keratin components possibly involved in inducing protease expression stays low. This emphasizes the need for autoclaving to enhance substrate availability for bacterial growth and enzyme production. Of note, washing was not identified as an essential pre-treatment step, since autoclaving alone is sufficient to achieve high feather degradation efficiency and high total protease activity. Finally, when looking at the impact of drying and grinding, they both seem to impact microbial hydrolysis for both strains in different ways.

The significant differences in protease activity between *B. licheniformis* and *A. oryzae* suggest that these bacteria exhibit distinct mechanisms of keratin degradation, possibly due to differences in enzyme secretion, regulation, or substrate specificity. The much higher protease activity of *B. licheniformis* indicates that this strain has a more performant proteolytic system capable of efficiently degrading complex protein substrates like keratin. This finding aligns with the observed overall degradation efficiencies and with previous research showing that *B. licheniformis* is a good keratinase producer [29, 34]. In contrast, *A. oryzae* displayed relatively low protease activity across the pre-treatments, with minimal variation in response to different feather preparations (Fig. 3). This suggests that rather than relying solely on high levels of protease production, *A. oryzae* could be utilizing a broader array of enzymes or mechanisms that effectively target different components of the feathers, which have not been described yet, leading to efficient degradation.

Specific keratinase activity

The results of the keratinase activity assay using azo keratin revealed notable differences between *A. oryzae* and *B. licheniformis* in their response to various feather pretreatments (Fig. 4). *B. licheniformis* exhibited significantly higher keratinase activity across all conditions compared

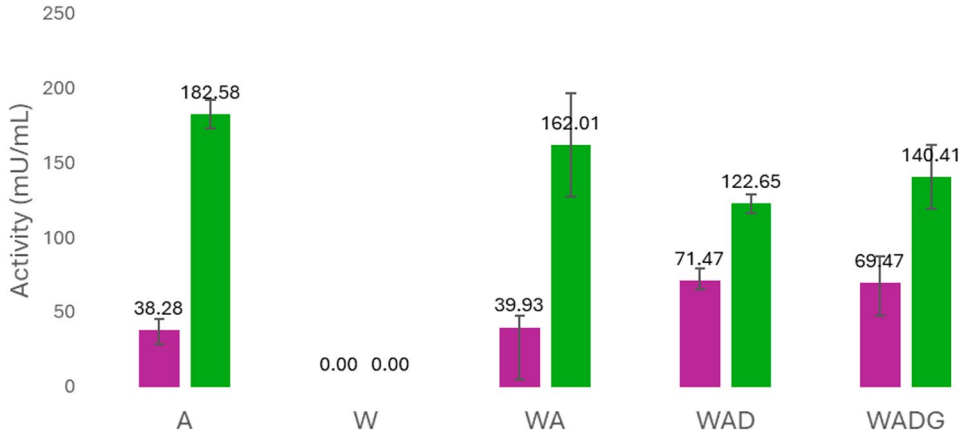

Fig. 4 Keratinase activity in culture supernatants after growing bacteria on feather materials. The feathers were pretreated by autoclaving (A), washing (W), washing and autoclaving (WA), washing, autoclaving and drying (WAD), and washing, autoclaving, drying, and grinding (WADG). The results are shown for 7 days of growth of *A. oryzae*(purple) and 5 days of growth of *B. licheniformis* (green). The standard deviation was calculated between the biological triplicates and technical duplicates

to *A. oryzae*. The highest activity was found when autoclaved (A) feathers were used, with a value of 182 mU/mL, followed by the WA condition at 162 mU/mL. For *A. oryzae*, the highest keratinase activity was observed for feathers treated by WAD, 71 mU/mL, and activity levels in the other cultures varied from 38 to 69 mU/mL. Neither of the two bacteria showed detectable keratinase activity in the cultures with washed treatment (W), well in line with the low non-specific activity shown in Fig. 3 and the notion that the autoclave treatment seems to be needed to induce protease production. Drying had a positive effect on this activity for *A. oryzae* while having a slightly negative impact for *B. licheniformis*.

It is not easy to rationalize the impact of varying feather pre-treatments on protease profiles. As alluded to above, such effects may relate to different factors that may affect gene expression in the bacteria, as well as different enzyme adsorption influenced by the pre-treatment method.

The keratinase activity data correlate well with degradation efficiency for both strains. Generally, the keratinase activities showed moderate differences between *A. oryzae* and *B. licheniformis*, which correlate with moderate differences in degradation efficiency. The ratio of keratinolytic to proteolytic activity has been proposed to reflect enzyme affinity for keratin [35]. In our previous study [38], this ratio was already reported to be higher for *A. oryzae* than *B. licheniformis.* Similarly, in this study, the same difference was observed in the ratio determined by dividing the keratinolytic activity (azo-keratin) by the non-specific proteolytic activity (azocasein). *A. oryzae* reached higher ratio values than *B. licheniformis* in all the treatments, with the highest difference found in WADG hydrolysates, with values of 1.58 and 0.14, respectively (Additional files 1, Fig. S2).

These results clearly show that the two strains have very different proteolytic machineries and may be taken to suggest that (1) the keratinase assay gives a good indication of actual keratinolytic potential, and (2) the proteolytic machinery of *A. oryzae* is more specific for keratin, compared to that of *B. licheniformis*.

Peptides profile analyzed by SEC

The hydrolysates were characterized by SEC to study the distribution of peptides produced by the two strains, and to examine the influence of different pre-treatments on the peptide profile. Although samples were standardized for a fixed concentration of dried material, the UV trace of the washed (W) feathers hydrolysates showed very low intensity. This is likely due to a much lower content of proteinaceous material than inorganic UV non-absorbent material. This correlates with the low degradation efficiency observed for these feather pre-treatments. Therefore, these hydrolysates were excluded from the analysis. To facilitate the analysis, the chromatograms were divided into four fractions (Fig. 5 and additional files 1, Fig. S3) based on retention time. The areas of the fractions were expressed as percentages of total area of the respective chromatogram.

Both the choice of the bacterial strain and the type of pre-treatment affected the peptide profile. The hydrolysates produced by *A. oryzae* generally contained larger peptides compared to *B. licheniformis* (Fig. 5). This is shown by looking at the molecular weight average values (Fig. 5B) but also by the fact that F2 was the most abundant fraction in all *A. oryzae* hydrolysates, while F3 was prevailing in *B. licheniformis* hydrolysates. This finding corresponds well with the other results presented in this study, showing clear differences in the proteolytic capabilities of the two bacteria.

Fig. 5 Size exclusion chromatogram analysis of feather hydrolysates. The feathers were pretreated by autoclaving (A), washing (W), washing and autoclaving (WA), washing, autoclaving and drying (WAD), and washing, autoclaving, drying, and grinding (WADG). The results are shown for 7 days of growth of *A. oryzae* and 5 days of growth of *B. licheniformis*. **A**: peptides fraction by size distribution shown as the relative abundance of four fractions, with (F1, retention time 5.00–9.95 min, Molecular weight > 1420 g/mol, F2, 9.95–11.87 min, MW 1420–410 g/mol, F3, 11.87–14.00 min, MW 410–80 g/mol and F4, 14.00–20.00 min, MW < 80 g/mol); and **B**: average molecular weight of peptides in hydrolysates generated by *A. oryzae* (in purple) and *B. licheniformis* (in green). The standard deviation was calculated between the biological triplicates and technical duplicates

As for the impact of the pre-treatment procedure, Fig. 5 clearly shows that a drying step (WAD and WADG) increases the peptide length. However, it must be noted that similar values of proteolytic activity were found between the different hydrolysates (Fig. 3). Considering that WAD and WADG contained higher amounts of proteinaceous material (see next sections) but mostly the same proteolytic activity than the other pre-treatments, it is expected a more limited peptide size reduction due to the saturation of the enzymes.

Determination of free sulfites and thiol groups

The hydrolysates were analyzed for free sulfite ions and thiol groups. Sulfite ion production promotes sulfitolysis, facilitating the cleavage of keratin's disulfide bonds and releasing free thiol groups [16]. This mechanism is commonly observed in efficient keratin degradation by keratinolytic microorganisms [16]. This assay revealed notable differences between the two strains. Figure 6A shows the levels of free sulfites in the hydrolysates, which were not detected in any of the hydrolysates produced by *A. oryzae*. The absence of sulfites in *A. oryzae* hydrolysates was already previously reported in a previous study [38] and explained by the fact that the keratinolytic strategy of *A. oryzae* does not generate reducing chemicals such as sulfites [35]. In contrast, the strategy of *B. licheniformis* relies on the conversion of cysteine into sulfite to assist the sulfitolysis [31]. However, this strategy seems to be affected by the pre-treatment of the feathers. Free sulfites in hydrolysates produced by *B. licheniformis* were only detected in hydrolysates from feather materials that included a drying step (20.7 µM for WAD and 52.7 µM for WADG). It is worth noting that the cysteine-sulfite conversion is also a mechanism of *B. licheniformis* to alleviate the cysteine toxicity. Additionally, a free sulfite accumulation may indicate that production exceeds consumption, suggesting a more complete sulfitolysis.

Overall, hydrolysates obtained from *B. licheniformis* presented higher levels of thiols than *A. oryzae* (Fig. 6B). This analysis also highlighted the negative impact of both drying and autoclaving on thiol levels in hydrolysates produced by both bacteria. This divergence shows that

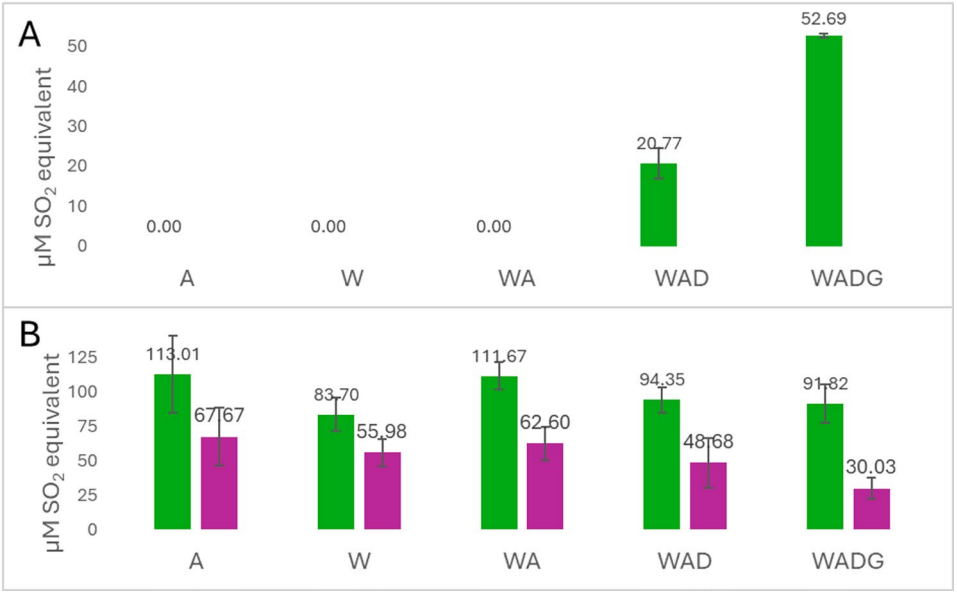

Fig. 6 **A**- Free sulfites and **B**- Thiol groups present in the hydrolysates after bacterial hydrolysis of feather materials. The feathers were pretreated by autoclaving (A), washing (W), washing and autoclaving (WA), washing, autoclaving and drying (WAD), and washing, autoclaving, drying, and grinding (WADG). The results are shown for 7 days of growth of *A. oryzae* (purple) and 5 days of growth of *B. licheniformis* (green). The standard deviation was calculated between the biological triplicates and technical duplicates

		Dry mass g/L	Total amino acid content g/L	Glu, E	Ser, S	Pro, P	Leu, L	Val, V	Arg, R	Gly, G	Asp, D	Cys, C	Ile, I	Thr, T	Phe, F	Ala, A	Tyr, Y	Lys, K	His, H	Met, M
				proportion of amino acid over total amino acid (%)																
	FM	10.00	8.21	12.45	11.03	8.56	8.20	7.26	7.11	6.90	6.61	6.32	5.06	4.91	4.87	3.97	2.75	2.38	0.92	0.71
	CM	13.09	9.64	12.81	10.68	8.35	8.15	7.29	6.97	6.80	6.82	5.96	5.13	4.89	4.89	4.09	2.76	2.72	1.04	0.68
A. oryzae	A	4.59	0.83	14.26	7.05	9.97	5.45	4.93	8.87	6.91	10.37	8.47	2.76	3.82	2.92	5.10	1.41	5.30	1.56	0.85
	W	2.74	0.21	12.87	7.21	7.31	7.21	6.87	10.11	6.43	8.66	3.35	4.82	5.97	3.98	6.75	0.00	5.32	1.91	1.25
	WA	3.48	0.57	14.28	6.68	10.54	5.36	4.78	9.66	6.62	10.63	8.63	2.48	3.70	2.76	4.96	1.21	5.11	1.67	0.92
	WAD	7.43	2.89	14.00	8.22	10.26	5.84	5.78	9.13	5.36	11.01	9.17	2.78	3.82	3.33	4.08	1.96	3.49	1.11	0.65
	WADG	8.35	3.63	13.58	8.76	11.43	6.53	6.20	8.53	5.71	9.86	8.42	3.93	3.85	3.15	3.60	1.70	3.05	1.07	0.63
	cell mass	--	--	19.51	4.33	3.76	7.38	6.34	7.31	4.78	8.18	0.40	4.06	5.98	3.28	10.00	1.78	8.35	2.20	2.37
B. licheniformis	A	5.65	1.15	7.65	4.61	7.96	3.92	3.43	7.17	3.96	7.91	8.58	1.80	2.70	15.67	2.66	8.12	9.82	3.34	0.70
	W	3.15	0.23	12.11	7.11	7.73	7.02	6.83	11.23	7.04	8.89	3.41	4.68	5.55	4.02	6.07	0.00	5.18	2.28	0.87
	WA	4.45	0.98	7.97	5.05	8.95	3.88	3.56	7.38	3.92	8.70	9.05	1.75	2.71	14.81	2.64	7.10	8.90	3.01	0.62
	WAD	10.07	3.29	8.49	6.00	8.90	4.19	3.60	7.24	3.52	10.66	9.55	1.57	2.91	14.07	2.50	7.79	5.63	2.42	0.97
	WADG	9.08	2.80	7.74	5.76	8.49	4.11	3.46	6.91	3.89	10.67	8.79	1.63	3.09	14.68	2.46	7.88	7.08	2.32	1.07
	cell mass	--	--	20.83	3.63	3.12	7.33	5.66	5.47	4.44	9.05	0.46	5.40	4.62	4.19	8.61	3.35	8.66	2.40	2.77

Fig. 7 Amino acid profile of the bacterial mass (obtained from BHI cultures), control medium (CM), feather meal (FM), and hydrolysates obtained from the microbial hydrolysis of feathers. The feathers were pretreated by autoclaving (A), washing (W), washing and autoclaving (WA), washing, autoclaving and drying (WAD), and washing, autoclaving, drying, and grinding (WADG). The results are shown for 7 days of growth of *A. oryzae* and 5 days of growth of *B. licheniformis*. The calculations were performed from the dry material of the hydrolysates and brought back to the proportions in the total amino acid content of each hydrolysate. With Glutamic acid (Glu, E), Aspartic acid (Asp, D), Proline (Pro, P), Arginine (Arg, R), Phenylalanine (Phe, F), Cysteine (Cys, C), Serine (Ser, S), Lysine (Lys, K), Leucine (Leu, L), Glycine (Gly, G), Valine (Val, V), Tyrosine (Tyr, Y), Threonine (Thr, T), Isoleucine (Ile, I), Histidine (His, H), and Methionine (Met, M)

while drying promotes sulfitolysis, it may also inhibit the subsequent release or stability of thiol groups. The obtained results imply that drying modifies keratin structure, possibly reducing *A. oryzae's* efficiency in processing disulfide-cleaved products into free thiols.

Overall, these findings indicate once more that *B. licheniformis* and *A. oryzae* apply different keratin degradation strategies. *B. licheniformis* relies primarily on sulfite-assisted sulfitolysis, while *A. oryzae* appears less dependent on sulfitolysis for disulfide bond cleavage, with no sulfites and fewer thiol groups present in the media. It also shows the greater impact of drying of the feather material on *B. licheniformis* feather hydrolyzation strategy.

Total amino acids of the hydrolysates

The analysis of total amino acids from the feather hydrolysates revealed the influence of both pre-treatment methods and bacterial strain (Fig. 7).

Overall, the hydrolysates were characterized by a lower amount of total amino acids than in the original feather material and control medium (Fig. 7). Both autoclaving and drying seemed to impact the total amino acid content of the hydrolysates, increasing it by up to five times for both bacteria.

When looking at the feather meal composition, glutamic acid (Glu), serine (Ser), proline (Pro), and leucine (Leu) are the amino acids present in the highest quantities. Due to the crucial role of disulfide bonds in the resistance and hardness of keratin, cysteine (Cys) was also present in considerable amounts (6%), which classifies feathers as hard keratin [32]. Interestingly, the hydrolysates produced by both bacteria showed elevated percentage of cysteine (Cys) in the amino acid profile. However, cysteine is not an essential amino acid for cell growth, and high concentrations can cause cytotoxicity [31]. The slightly higher percentage of cysteine in the hydrolysates is probably due to the fact that both bacteria consume other amino acids over cysteine, and consequently, its proportion is enriched. This is confirmed by the concentration of cysteine in hydrolysates, which was still lower than the maximum marked by the initial control medium (0.31 g/L vs. 0.57 g/L) (Additional files 1, Table S2). Furthermore, WA and WAD treatment for *B. licheniformis* exhibited the highest cysteine concentrations, which correlates with a higher production of sulfites.

Other amino acids, such as phenylalanine (Phe), tyrosine (Tyr), and lysine (Lys), are present in high proportion in the hydrolysates obtained from *B. licheniformis*. To give an instance, the sum of these amino acids reached 27.5% of the total amino acid content in WAD hydrolysate. In this hydrolysate, these amino acids were in similar concentration as in the initial medium (CM): 0.46 g/L vs. 0.47 g/L for phenylalanine, 0.26 g/L vs. 0.27 g/L for tyrosine and 0.20 g/L vs. 0.26 for lysine (Additional files 1, Table S1). Note the difference in dry mass content between CM and WA (13.09 g/L vs. 10.07 g/L), since a total degradation was not achieved, and *B. licheniformis* biomass consumed significant amounts of these amino acids among other medium components. Therefore, the only explanation for these concentrations is the overproduction and secretion of these amino acids by *B. licheniformis*.

Secretion of these amino acids in amounts significantly higher than necessary for protein synthesis has already been reported for other species from the *Bacillus* genus [8, 13, 28]. The secretion of these amino acids from non-feed protein waste is industrially relevant [18]. To the authors' knowledge, this study is the first one reporting hypersecretion of phenylalanine, tyrosine, and lysine in *Bacillus licheniformis*. Moreover, this outlines the benefit of microbial hydrolysis over enzymatic hydrolysis, since it can enrich the hydrolysates in target amino acids using the appropriate amino acid secreting species.

Elemental analysis

The micro and macro element composition of the hydrolysates, control medium (CM), and feather meal is presented in Fig. 8a, along with the ratio of nitrogen to carbon (N/C).

The most noticeable observation comes when comparing the N/C ratio of the hydrolysates of both bacteria with the initial reference medium (CM). A higher N/C ratio was found in all hydrolysates regardless of the pre-treatment used. This can be explained by the fact that the feather meal, as a sole source of nitrogen and carbon, exceeds the physiological nitrogen requirements of both bacteria, as discussed in our previous study [38]. The bacteria consume carbon proportionally more than nitrogen according to their needs, resulting in a nitrogen enrichment of the hydrolysates.

When looking deeper, some differences in the N/C ratio were found also between the different pre-treatments. It seems that for both bacteria hydrolysates, there is a negative correlation between the dry mass content and the N/C ratio. For instance, W hydrolysates reached values around 0.58–0.60 N/C and 2.74–3.15 g/L dry mass content, while WADG hydrolysates reached values of around 0.37 N/C and 8.35–9.08 g/L dry mass content for both bacteria. This suggests a certain loss of nitrogen when an efficient hydrolysis treatment is used. As previously explained, feather meal exceeds the nitrogen required by the bacteria. Consequently, the bacteria perform an extensive deamination of the proteinaceous materials to fulfill the higher carbon than nitrogen requirements. Deamination also explains the mismatch between the values of total amino acids and protein estimation using the nitrogen content and the x6.25 Jones conversion factor (e.g., *A. oryzae* WADG 3.63 g/L vs. 5.43 g/L). As a result of deamination, the exceeding nitrogen is secreted in the form of NH_3. In our previous study [38], the hydrolysis process of feather meal by these two bacteria was monitored daily, showing an increase in NH_3 and its progressive evaporation from the culture. Assuming that the NH_3 release was faster and earlier in the most efficient conditions (WAD and WADG), it can be expected that its evaporation occurred earlier, too. Thus, the more efficient the treatment, the greater the amount of NH_3 was proportionally lost, leading to the N/C differences observed between hydrolysates.

On the other hand, sodium and phosphate seemed to be similar or higher in the hydrolysates than in the control medium. These increased levels of salts can be linked to the bacterial inoculum grown in BHI broth, that contains animal materials alongside disodium hydrogen phosphate and sodium chloride in considerable amounts.

A		Dry mass (g/L)	N/C	C	N	S	Ca	P	Mg	Na	K
				(g/L)							
	FM	10.00	0.29	4.93	1.44	0.20	0.01	0.01	0.00	0.00	0.00
	CM	13.09	0.29	6.04	1.74	0.49	0.05	0.05	0.21	0.01	0.07
A. oryzae	A	4.59	0.45	0.93	0.42	0.41	0.03	0.04	0.24	0.09	0.10
A. oryzae	W	2.74	0.60	0.29	0.17	0.33	0.03	0.01	0.18	0.10	0.07
A. oryzae	WA	3.48	0.49	0.63	0.30	0.33	0.03	0.03	0.20	0.07	0.08
A. oryzae	WAD	7.43	0.38	2.28	0.87	0.49	0.03	0.03	0.23	0.08	0.08
A. oryzae	WADG	8.35	0.37	2.84	1.05	0.49	0.03	0.02	0.20	0.08	0.09
B. licheniformis	A	5.65	0.41	1.34	0.55	0.42	0.04	0.04	0.24	0.08	0.09
B. licheniformis	W	3.15	0.58	0.35	0.20	0.33	0.03	0.01	0.18	0.09	0.08
B. licheniformis	WA	4.45	0.43	1.05	0.45	0.35	0.03	0.03	0.20	0.07	0.07
B. licheniformis	WAD	10.07	0.39	3.07	1.19	0.61	0.05	0.03	0.30	0.09	0.10
B. licheniformis	WADG	9.08	0.37	2.77	1.02	0.57	0.03	0.04	0.29	0.08	0.09

B		Dry mass (g/L)	Fe	Zn	Cu	Mn
			(mg/L)			
	FM	10.00	0.49	1.12	0.15	0.04
	CM	13.09	3.28	1.48	0.17	0.08
A. oryzae	A	4.59	0.80	0.23	0.01	0.02
A. oryzae	W	2.74	0.31	0.09	0.14	0.00
A. oryzae	WA	3.48	0.69	0.19	0.12	0.01
A. oryzae	WAD	7.43	0.98	0.53	0.50	0.02
A. oryzae	WADG	8.35	0.62	0.50	0.07	0.04
B. licheniformis	A	5.65	1.28	0.43	0.03	0.00
B. licheniformis	W	3.15	0.67	0.13	0.22	0.00
B. licheniformis	WA	4.45	0.68	0.32	0.09	0.00
B. licheniformis	WAD	10.07	1.27	0.76	0.69	0.00
B. licheniformis	WADG	9.08	0.99	0.53	0.06	0.00

Fig. 8 Elemental composition of A: macro elements and B: micro elements present in the hydrolysates, control medium (CM), and feather meal (FM). With macro elements: phosphorus (P), calcium (Ca), potassium (K), sodium (Na), magnesium (Mg), sulfur (S), nitrogen (N) and carbon (C) and micro elements (manganese (Mn), copper (Cu), zinc (Zn), and iron (Fe). The hydrolysates were obtained from the hydrolysis of feather pretreated with different methods: autoclaving (A), Washing (W), washing and autoclaving (WA), washing autoclaving and drying (WAD), and washing autoclaving drying and grinding (WADG). The results are shown for 7 days of growth of *A. oryzae* (purple) and 5 days of growth of *B. licheniformis* (green)

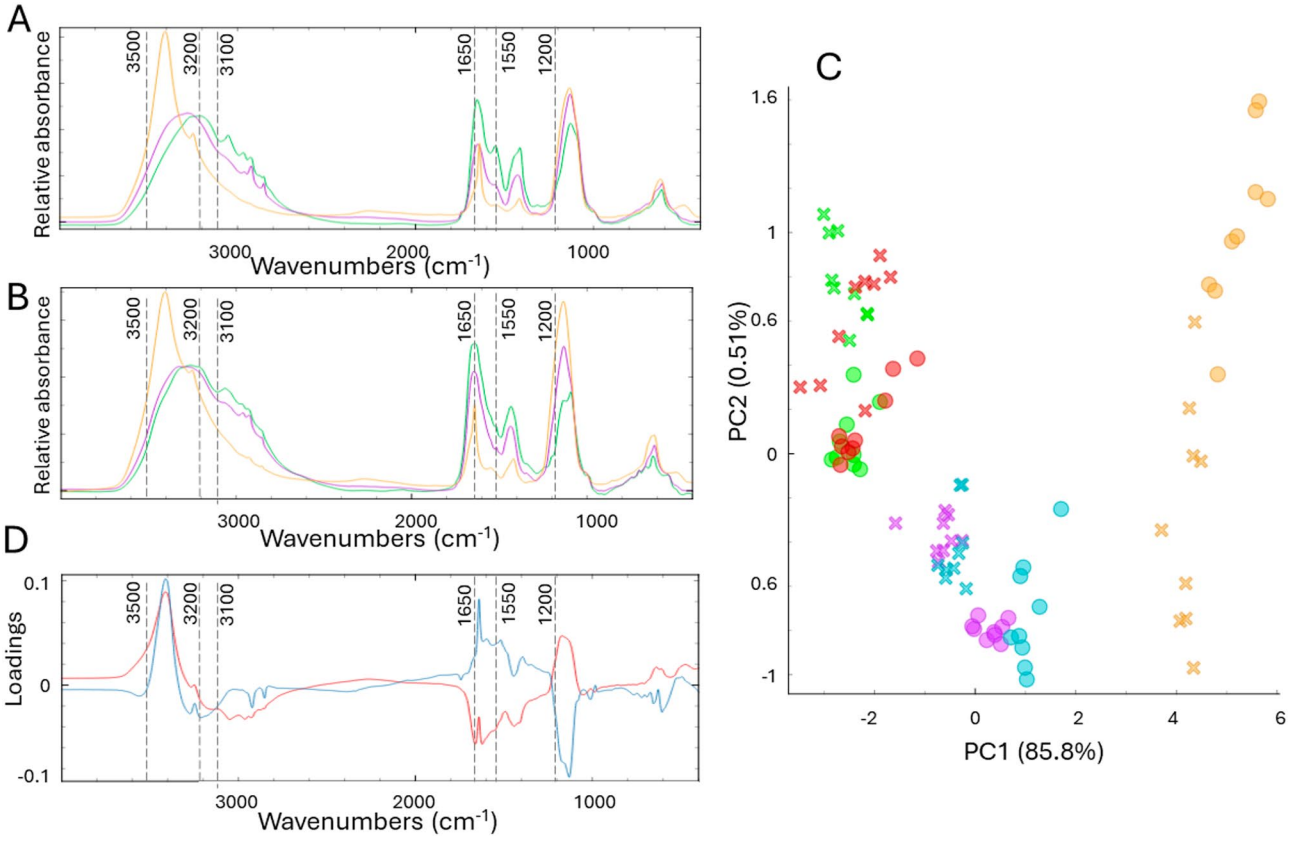

Fig. 9 Preprocessed FTIR-HTS spectra and the plots of the PCA on the feather microbial hydrolysates FTIR spectra. Preprocessed FTIR spectra of hydrolysates were obtained using (**A**) *A. oryzae* and (**B**) *B. licheniformis*, averaged between triplicates. The spectra are colored according to the pre-treatment conditions: autoclaving (A; purple), washing (W; orange), washing, autoclaving, drying, and grinding (WADG; green). (**C**) PCA scores plot, colors by pre-treatment conditions: A – purple, W – orange, WA – turquoise, WAD – red, WADG – green) and shapes by bacterial strain (round: *Arthrobacter oryzae*; cross: *Bacillus licheniformis*). Each point represents a single hydrolysate sample, and the distance between points reflects similarity or dissimilarity of the peptide profiles. (**D**) PCA loadings plot of PC1 in red and PC2 in blue. The percent variances for the first five PCs are 85.8, 0.51, 0.28, 0.24, and 0.12

Total biochemical profile by FTIR spectroscopy

FTIR spectroscopy is a powerful analytical technique employed to obtain information about the molecular composition and functional groups of various substances, such as hydrolysates [25, 46]. FTIR spectroscopy is a key approach in biochemistry for studying protein structures, evaluating functional group changes, and assessing the chemical composition of hydrolysates and other complex mixtures [41]. It has already been used for monitoring protein hydrolysis [41] and characterizing protein hydrolysates [25]. Recent studies have also utilized FTIR for tracking chemical changes in feather hydrolysis [38, 46]. In this study, FTIR was used to explore differences and similarities in total biochemical composition of the hydrolysates.

Figure 9A and B show HTS-FTIR spectra of the hydrolysates from both *A. oryzae* (A) and *B. licheniformis* (B). Spectra from washed and autoclaved (WA) feathers and washed autoclaved and dried (WAD) feathers are very similar to the spectra of autoclaved (A) feathers and washed, autoclaved, dried, and grinded (WADG) feathers, respectively (the figure with all spectra is available in the additional file 1, Fig. S3). The main peaks of interest are related to amide groups: amide A and B N-H stretching vibrations (approx. 3300 cm^{-1} and 3100 cm^{-1}, respectively), O-H stretching vibrations (3500–3200 cm^{-1}), amide I C = O stretching vibrations (approx. 1650 cm^{-1}), amide II band (1550 cm^{-1}), and amide III (around 1200 $^{-1}$).

PCA of all FTIR spectra (Fig. 9C) reveals that hydrolysates from both bacterial strains appear to cluster into three distinct groups, with the separation occurring along both PC1 and PC2. The difference between the non-autoclaved samples and all other samples is represented by PC1, as visible by the loadings (Fig. 9D), with non-autoclaved samples having higher PC1 scores than autoclaved samples. In a minor way, PC1 is also separating the dried samples, though the drying step is mostly represented by PC2 (Fig. 9D), with dried samples having higher PC2 values than non-dried samples. Moreover, the influence of strains on all samples is also represented by PC2, with *B. licheniformis* samples having higher PC2 values than the

corresponding *A. oryzae* samples. The only exception is non-autoclaved samples, where this pattern is reversed. In both PC1 and PC2, high positive factor loadings is associated with the 3500–3000 cm^{-1} region, specifically 3300 cm^{-1} and 3100 cm^{-1} peaks related to O-H and N-H stretching vibrations. The pattern related to the amides I, II and III band (1650 cm^{-1}, 1550 cm^{-1} and 1200 cm^{-1}) is different for PC1 (negatively associated) and PC2 (positively associated). This is likely related to changes in the protein structures, indicated by bands in amide I and III regions, related respectively to α-helical structures (more present in dried samples) and β-pleated sheet structures (more present in non-autoclaved samples). Finally, these treatments and the choice of strain also impact on the size of peptide fragments present in the hydrolysates, as suggested by the higher ratio of amide II to amide I band increased by autoclaving and drying.

Overall, the FTIR spectra highlight the significant impact of processing steps on the chemical composition and structural characteristics of the bacterial feather hydrolysates, with both autoclaving and drying affecting the composition of the hydrolysates. Differences in bacterial strains once more point to varying feather degradation strategies from the two strains. *A. oryzae* hydrolysates showed signs of less complete degradation compared to those from *B. licheniformis*, which coincides with conclusions obtained from SEC and amino acid analysis, showing different profiles for the two bacteria as well as for autoclaving and drying of feathers.

Conclusions

Microbial hydrolysis of feathers, using each of the bacterium tested in this study, is efficient. This is highlighted by high degradation efficiency and furthermore, by much higher ratios of nitrogen to carbon in all hydrolysates compared to the starting feather meal, resulting in an increased industrial value of the product.

The choice of bacterial strain significantly impacts the hydrolysis process and hydrolysate composition, highlighting differences in enzymatic strategies for keratinolysis. For example, *Bacillus licheniformis* CCM 2145^{T}, a bacterium widely used in industrial applications [3, 44], exhibited higher protease activity and relied on sulfite-assisted sulfitolysis for keratin degradation. The hydrolysates produced by this strain contained smaller, bioactive peptides, making them suitable for pharmaceutical or cosmetic applications. Additionally, this model organism demonstrated higher thiol levels across all pretreatments, reflecting its efficient disulfide bond cleavage mechanisms. In contrast, *A. oryzae*, the newly isolated cold-adapted bacterium [38] tested in this study, showed lower protease activity but achieved effective degradation through a more specialized keratinolytic strategy. The hydrolysates derived from *A. oryzae* contained larger peptides, which are potentially suitable for sustained protein release in feed or food applications. Interestingly, *A. oryzae* was less sensitive to variations in pre-treatment conditions, maintaining consistent degradation efficiency.

Pre-treatment plays a crucial role in optimizing microbial feather degradation and hydrolysis. Autoclaving enhances the degradation efficiency greatly by disrupting the structural integrity of feathers, making keratin more accessible and enhancing bacterial enzyme activity. Additionally, it alters the overall composition of hydrolysates, leading to the formation of hydroxyl, carbonyl, and amide functional groups, as observed in FTIR spectra. Drying appears to slightly reduce degradation efficiency by altering keratin structure, denaturing proteins, and influencing peptide size and thiol stability, especially in *A. oryzae* BIM B-1663. Nevertheless, it has a significant impact on hydrolysate composition, particularly regarding nitrogen to carbon ratio and amino acid content. Hydrolysates from both bacterial strains exhibit higher amino acid concentrations in samples containing dried feathers. Furthermore, *B. licheniformis* seems to modify the amino acid profile of the hydrolysate in comparison with the initial medium. Indeed, the hydrolysates from *B. licheniformis*, especially using dried and dried and grinded feathers, are enriched in amino acids of commercial interest (phenylalanine, lysine, and tyrosine) [18].

Overall, optimizing and scaling up feather hydrolysis processes using *A. oryzae* BIM B-1663 could enable more energy-efficient production of feather hydrolysates. Using this strain with dried feathers enables efficient feather degradation while producing hydrolysates with a rich amino acid composition and especially high content in nitrogen, holding promise for application in feed and fermentation industry.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02743-8.

Supplementary Material 1

Supplementary Material 2

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Author contributions

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Data availability
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