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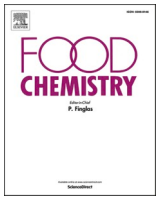

# Beneficial effect of oat-based postbiotics produced through capsule or free-cell fermentation on lipopolysaccharide activated intestinal cells

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ABSTRACT

The aim of this study was to design an innovative oat fermentation process using capsules to produce postbiotics with anti-inflammatory and antioxidant potential. Capsule fermentation led to higher bacterial growth ($3.30*10^9$ CFU/mL) and lactic acid production (20.49 mg/mL), compared to traditional free-cell fermentation ($4.73*10^8$ CFU/mL and 9.08 mg/mL, respectively), along with increased cell and product yields. The dried postbiotics were also subjected to in vitro digestion, mimicking the gastrointestinal one. A functional characterization of the four postbiotics, undigested and digested postbiotics from both capsule and free-cell fermentation, was performed using the HCT116 colon cell line. All four products prevented LPS- induced activation of the inflammatory pathway, protein oxidative damage, and impaired glutathione reductase activity, with digested postbiotics being more effective than undigested ones. These findings demonstrate that the capsule-based postbiotic is a promising candidate as a functional food with potential applications in nutraceuticals, animal feeding, and cosmetics.

## 1. Introduction

The consumption of unhealthy diets, such as Western diets (WD), rich in processed food, saturated fatty acids, sugar, and red meats, is strongly associated with the incidence of chronic diseases, including obesity, cardiovascular disease (CVD), and type 2 diabetes mellitus (Clemente-Suárez et al., 2023). The report published by the EAT-Lancet Commission highlighted the importance of shifting to healthy diets consisting of low amounts of animal-source foods, unsaturated rather than saturated fats, and a diversity of plant-based foods, promoting not only human health but also environmental benefits (Willett et al., 2019).

Oats (*Avena sativa L.*) are considered one of the healthiest and most sustainable foods worldwide. Consumption of oat products has been associated with reduced cholesterol levels, lower risk of cardiovascular disease, obesity, cancer, diabetes, and gastrointestinal disorders (Joyce et al., 2019). Oats are rich in different components (indigestible oligosaccharides, essential amino acids) and bioactive compounds (such as beta-glucans, bioactive peptides, polyphenols, and avenanthramides), which makes them a promising food matrix for microbial growth, biological activity (Gupta & Bajaj, 2018), and postbiotics production (Yang et al., 2023). Fermented oats can increase the contents of non-nutritive phytochemicals, including phenolic acids, avenanthramides, and

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flavonoids, which have been associated with various health benefits, including anti-inflammatory and antioxidant activities, which could potentially reduce the risk of non-communicable diseases (Djorgbenoo et al., 2023).

Postbiotics, which are a step beyond probiotics, are recently emerging as a valid alternative to live microbial cells, offering stability, safety, and avoiding the risk associated with live microorganisms (Amobonye et al., 2025). They are defined as preparations of inanimate microorganisms and/or their components that confer a health benefit on the host (Salminen et al., 2021), and are generally obtained through a fermentation process followed by a subsequent heat treatment, which inactivates microorganisms and provides a safer profile compared to probiotics (Amobonye et al., 2025; Salminen et al., 2021). The fermentation process is typically conducted in a batch reactor at an industrial scale. Reducing the reaction volume by immobilizing microorganisms through encapsulation can improve the process by minimizing spatial inhomogeneities. Capsules used as mini-bioreactors ensure a more controlled and efficient process, overcoming some limitations associated with traditional free-cell methods (Mitropoulou et al., 2013), by reducing the non-productive growth phase and obtaining a higher cell density than free-cell fermentation, leading to increased cell productivity and higher product yields (Kurzbaum et al., 2020; Oliveira et al., 2011; Ramakrishna & Prakasham, 1999). Also, encapsulation prevents the degradation of functional compounds during storage, ultimately ensuring their effective absorption into the body after digestion (Alonso et al., 2015; Sathyabama et al., 2014). Various methods, including spray drying, coacervation, and extrusion, are employed to design capsules (Bamidele & Emmambux, 2021; No & Shin, 2019; Zhao et al., 2019). Extrusion, a conventional, simple, and cost-effective technique, is particularly useful because it minimizes loss of trapped bioactive components or probiotics (Bamidele & Emmambux, 2021). The screening of encapsulating materials and the optimal bacterial growth substrate are key factors in the encapsulation and fermentation processes, as well as the substrate undergoing fermentation. In this context, oats, with their natural bioactive molecules, emerge as a promising substrate. To the best of our knowledge, no other study has examined the use of an oat-based postbiotic derived from fermentation in capsules.

It was hypothesized that encapsulation could optimize fermentation and protect the functional components produced, thereby enhancing their effects. Indeed, starch in oats plays an important role as an excellent carrier material for encapsulated probiotics, protecting them from environmental factors and ensuring their long-term efficacy (Shah et al., 2016). Therefore, this study aims to investigate whether encapsulation improves fermentation efficiency using *Lacticaseibacillus paracasei CBA L74,* already known for its postbiotic potential (Corsello et al., 2017; Gallo et al., 2019; Roggero et al., 2020; Sarno et al., 2014), and whether postbiotics obtained by this approach exert anti-inflammatory effects.

To fulfill this aim, the postbiotic obtained using capsules as mini-bioreactors was characterized, and the fermentation efficiency of this innovative method was compared with that of conventional free-cell fermentation. The products obtained from the two processes were compared for their potential anti-inflammatory activity in a model of lipopolysaccharide (LPS)-activated intestinal cells, both as such and after an in vitro digestion process mimicking gastrointestinal digestion.

## 2. Materials and methods

### 2.1. Materials

Bacto Yeast Extract was purchased from BD Biosciences (Milan, Italy); oat flour (Whole oat flour, Le Farine magiche, Lo Conte Group) was purchased from a local market; MRS agar and MacConkey agar were purchased from Oxoid (Basingstoke, UK); Gelatin Peptone Bios Agar was purchased from Biolife (Milan, Italy). D-glucose assay kit GOPOD format and *E*-BLAAM amylase were purchased from Megazyme (Bray, Ireland); Amicon filters used for dialysis had a 3 kDa cut-off and were purchased from Merck Life Science S.r.l. (Milan, Italy). The following products were purchased from Sigma-Aldrich (St. Louis, MO, USA): magnesium sulfate ($MgSO_4$), glucose, citric acid, sodium alginate (from brown algae), calcium chloride ($CaCl_2$), sodium chloride (NaCl), sodium citrate, potassium dihydrogen phosphate ($KH_2PO_4$), phosphoric acid ($H_3PO_4$), salivary amylase 1031, hydrochloric acid (HCl), porcine pepsine P6887, sodium hydroxide (NaOH), porcine pancreatine P7545, bile salts B8631, Lypopolysaccharide (LPS) from *E. coli*, (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), orthophenylenediamine dichloride (OPD), hydrogen peroxide, mouse monoclonal antibody (AC-74) to beta-actin, rabbit polyclonal antibody to TLR4. Rabbit polyclonal antibody to calnexin was supplied by Abcam (Cambridge, UK). Rabbit polyclonal antibodies to MyD88, phospho-NFkB, NFkB, phospho-eIF2α and eIF2α were purchased from Cell Signaling (MA, USA). Rabbit polyclonal antibody to nitro-Tyrosine (N-Tyr; CVL-PAB0188) was from SIAL (Rome, Italy). Fuji Super RX 100 films photographic plates, development and fixing reagents come from the Precision Electronic Laboratory (Naples, Italy). The Excellent Chemiluminescent Kit (Elabscience), goat anti-rabbit horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA), and ELISA microtiter plates (Nunc MaxiSorp) were purchased by Microtech (Naples Italy). Nitrocellulose filters (0.45 and 0.22 μm) to filter the solutions were purchased from the company Merk Millipore (Milan, Italy). Disposable material, salts, molecular weight markers, bovine serum albumin (BSA), organic solvents were purchased from the company Del-Tek (Naples, Italy). The nitrocellulose membrane for the transfer of proteins into the electrical field was purchased by the company GE Healthcare (Milan, Italy). The culture medium RPMI, L-Glutamine, Penicillin, Streptomycin, Fetal Bovine Serum (FBS), Trypsin, cell culture flasks (25 $cm^2$), Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail, cell culture plates of 6 and 92 wells, conical polypropylene tubes of 15 and 50 mL, microtubes of 1.5 mL and sterile pipettes were purchased from Euroclone (Milan, Italy).

### 2.2. Development of the Postbiotic dry products

#### 2.2.1. Fermenting strain

For the fermentation process, the strain *Lacticaseibacillus paracasei CBA L74*, provided by Heinz Italia S.p.A. and deposited in BCCM (International Depository Accession Number LMG P-24778), was utilized. This microorganism, isolated from the feces of a healthy child, is gram-positive and homofermentative. It was stored at −80 °C using 20% glycerol. Both the storage and revitalization medium consisted of an animal-free broth made with the following components: 20 g/L Bacto Yeast Extract, 0.5 g/L $MgSO_4$, 50 g/L glucose, and 0.5 g/L citric acid. The revitalization process involved adding 9 mL of the animal-free broth and incubating the mixture at 37 °C for 24 h, resulting in a bacterial concentration close to $10^8$ CFU/mL.

#### 2.2.2. Preparation of the oatmeal suspension used as fermenting and encapsulating material

A hydrolyzed oatmeal suspension was used both as a fermentation substrate and as an encapsulation material. The suspension was prepared with 15% *w/v* oat flour in water, supplemented with 1% w/v glucose. The chemical composition of the oat flour is reported in the supplementary Table 1. The oatmeal suspension was pretreated as previously described (Lentini et al., 2022), using a multifunction stainless-steel machine (Hotmixpro Twin Cutter) with an anchor impeller and a motor power of 1500 W. Briefly, 0.054% w/v amylase in water was activated by heating at 75 °C for 20 min. Following enzymatic activation, oat flour (15% *w/v*) and glucose (1% w/v) were incorporated. Further steps of 75 °C for 25 min and 90 °C for 20 min were carried out to ensure complete starch gelatinization and optimal enzyme activity within the grains. Finally, the sterilization cycle was performed in an autoclave at 134 °C for 40 min.

#### 2.2.3. Encapsulation protocol

Capsules were produced as previously described (Passannanti et al., 2024). Briefly, a 1% w/v sodium alginate solution was combined with a hydrolyzed oatmeal suspension prepared as described in section 2.2.2, and mixed until a homogeneous consistency was achieved. Revitalized L. *paracasei CBA L74* was added to achieve a final bacterial concentration of $10^6$ CFU/mL, followed by gentle mixing. The resulting mixture was then dispensed using a peristaltic pump (Watson Marlon Alitea SciQ 400) equipped with a 2 mm inner diameter silicone tube, at a flow rate of 5 mL/min. Droplets were formed by releasing the mixture from a height of 10 cm into a 1 M calcium chloride solution and allowed to set for 20 min.

#### 2.2.4. Fermentation apparatus

The system comprised a batch reactor measuring 20 cm in height and 10 cm in internal diameter, with a total volume of 1.5 L. It was designed with an external jacket to circulate a service fluid (water) from a precisely controlled thermostatic water bath (Haake G) to maintain temperature. Mixing was achieved with a stainless-steel rotating shaft mounted on the head plate fitted with two Rushton turbines. The motor-driven shaft allowed precise adjustments of stirring speed, ensuring uniform medium homogeneity throughout fermentation. The head plate was also equipped with an entry port for an In Pro 3100 probe (Mettler Toledo, Milan, Italy), seamlessly connected to an M300 transmitter (Mettler Toledo, Milan, Italy) for reliable inline temperature and pH monitoring.

#### 2.2.5. Free-cell and capsule fermentation protocols

Two distinct fermentation processes were performed: free-cell fermentation and capsule fermentation. Both processes shared several steps, including pre-treatment and sterilization of the hydrolyzed oatmeal suspension, sterilization of the fermentation apparatus, and revitalization of the microbial strains. In the free-cell fermentation, the microorganism, after a 24-h revitalization, was directly inoculated into a reactor containing 1 L of the hydrolyzed oatmeal suspension, and the fermentation proceeded at 37 °C for 24 h. For capsule fermentation, following the same revitalization period, the microorganism was encapsulated as previously described. The resulting capsules were suspended in hydrolyzed oatmeal suspension at a fixed ratio of 1:3 (capsule volume to external liquid volume), and the fermentation proceeded at 37 °C for 24 h.

At the end of fermentation, the products from both free-cell and capsule fermentation were thermally treated (85 °C for at least 1 min) to inactivate the bacterial charge, then subjected to drying to obtain the final postbiotics. The free-cell postbiotic was freeze-dried (Christ, Alpha 1–2 LDplus), while capsules, after being filtered from the medium, were dried by an air dryer (37 °C for 4 h).

A scheme of the experimental design is reported in the Supplementary Fig. 1 to better clarify the adopted procedures.

### 2.3. Characterization of the fermentation process

#### 2.3.1. Bacterial growth

Aseptic sampling was performed at the start of the process (t0) and after 2, 4, 6, 16, 18, 20, 22, and 24 h. Samples from free-cell fermentation were serially diluted in 0.9% NaCl. Capsules collected from the capsule fermentation required a preliminary phase: capsules were broken in sodium citrate solution (1% *w*/w, pH 6.0) at 1:10 ratio (capsule weight to sodium citrate volume). After disruption, the capsules were serially diluted as previously described for samples from free-cell fermentation.

The samples were then spread-plated on MRS agar plates for the enumeration of Lactobacilli and on MacConkey agar and Gelatin Peptone Bios Agar to verify the potential presence of microbial contaminants.

Plates were incubated at 37 °C for 48 h.

#### 2.3.2. Lactic acid production

To analyze lactic acid production, samples from the two processes were pretreated to allow fiber separation. Each sample was mixed with 50 mM phosphate buffer at 1:3 ratio, gently stirred at 40 °C for 1 h, and finally centrifuged at 10,000 *g* for 10 min. The supernatant was collected and filtered through a 0.45 μm cutoff filter. Capsules were first broken in a sterile solution of 1% (*w*/w) of sodium citrate at pH 6, using a 1:10 ratio (capsule weight to solution volume) before pretreatment. Lactic acid analysis was carried out by HPLC using an Agilent 1100 series instrument (Agilent Technologies, Milan, Italy), equipped with a visible/UV detector and a Synergi 4 μm Hydro-RP 80 Å column. The mobile phase consisted of a 0.27% (*w/v*) aqueous $KH_2PO_4$ solution at pH 1.5, adjusted with $H_3PO_4$. The column temperature was maintained at 60 °C with a flow rate of 1 mL/min, and detection was set at 210 nm. Lactic acid content was quantified by using a calibration curve with lactic acid as a standard (50–1000 mg/L).

#### 2.3.3. Glucose consumption

Glucose consumption during fermentation was assessed using the D-glucose assay kit, GOPOD format. Prior to analysis, samples underwent pretreatment. Free-cell samples were diluted 1:20 with distilled water, while capsule samples were diluted 1:2. Both sample types were sonicated with a Bandelin Sonorex sonicator at 35 kHz for 15 min. After sonication, the samples were centrifuged at 10,000 *g* for 10 min and filtered through a 0.45 μm filter before analysis.

#### 2.3.4. Cell and product yields

Once obtained bacterial growth, lactic acid production and glucose consumption, two different yields were calculated according to the following Equations:

$$Cell\ Yield = \frac{\Delta bacterial\ growth}{Substrate\ consumption} = \frac{(t_{24}g\ bacteria) - (t_0\ g\ bacteria)}{(t_0\ g\ glucose) - (t_{24}g\ glucose)} \quad (1)$$

$$Product\ yield = \frac{Lactic\ acid\ production}{Substrate\ consumption} = \frac{(t_{24}\ g\ lactic\ acid)}{(t_0\ g\ glucose) - (t_{24}g\ glucose)} \quad (2)$$

To obtain the g of bacteria, a conversion factor ($1.67*10^{-12}$ g/CFU), previously calculated for *Lacticaseibacillus paracasei CBA L74*, was used.

#### 2.3.5. Kinetic parameters

After obtaining the bacterial growth curve, the doubling time (td), which represents the time required for the bacterial population to double during the exponential growth phase, was calculated using Eq. 3 (Maier, 2009).

$$t_d = t_{exp}/n \quad (3)$$

In this equation, $t_{exp}$ refers to the duration of the exponential growth phase, while n represents the number of generations. The number of generations is determined using Eq. 4 (Maier, 2009), where $N_f$ and $N_0$ are the bacterial counts at the end and beginning of the exponential growth phase, respectively.

$$N_f = N_0 x\ 2^n \quad (4)$$

Additionally, the constant growth rate was calculated using Eq. 5.

$$k = n/t_{exp} = 1/t_d \quad (5)$$

The growth rate, denoted as $r_x$, can be expressed in terms of first-order kinetics with respect to biomass concentration, according to Eq. 6

$$r_x = dX/dt = \mu X \quad (6)$$

Where X represents the number or mass of cells (mass/volume), t is the time, and μ is the specific growth rate constant of the fermentation process (1/time). By applying the method of separation of variables to Eq. 5 and integrating with the initial condition ($X = X_0$ at $t = t_0$), Eq. 7

was obtained.

$$\mu = \frac{\ln\left(\frac{x}{x_0}\right)}{t - t_0} \quad (7)$$

### 2.4. Characterization of the postbiotic dry products

#### 2.4.1. In vitro digestion

Postbiotic dry products obtained by capsule and free-cell fermentation were subjected to in vitro digestion, following the INFOGEST protocol (Brodkorb et al., 2019). Briefly, the oral phase began by mixing the samples with the simulated salivary fluid (SSF) at a 1:3 ratio, containing human salivary amylase (75 U/mL). This ratio was determined based on preliminary tests to ensure complete coverage and interaction of the sample with the digestive fluid. The oral phase lasted 2 min. The gastric phase began by combining the bolus obtained from the oral phase with the simulated gastric fluid (SGF) in a 1:1 ratio. After mixing, the pH was quickly adjusted to 3 by adding 1 M HCl. Porcine pepsin was then introduced at a final concentration of 2000 U/mL. Gastric phase lasted 2 h, at 37 °C. Finally, during the intestinal phase, the chyme resulting from the gastric phase was mixed with simulated intestinal fluid (SIF), and the pH was quickly corrected to 7 using 1 M NaOH. The SIF contained porcine pancreatin, added to achieve a trypsin activity of 100 U/mL, as well as bile salts at a concentration of 10 mM. Intestinal phase was conducted for 2 h at 37 °C. Following the INFOGEST protocol, digested samples were dialyzed using Amicon filters with a 3 kDa cutoff (Brodkorb et al., 2019). In Table 1, a schematic description of the postbiotic samples subjected to analysis is shown.

#### 2.4.2. Total polyphenol content, Total flavonoid content, antioxidant activity

Undigested postbiotics dry products obtained by capsule (CAPpb), free-cell fermentation (FCpb), and digested postbiotic dry products from capsule and free-cell fermentation (dCAPpb and dFCpb) were analyzed for their Total Polyphenol Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Activity (AA). Polyphenols were extracted from all postbiotic samples: 0.5 g of each sample were combined with 7 mL of a 70% *v*/v ethanol solution, sonicated at 35 kHz for 15 min, and centrifuged at 2000 rpm (430 *g*) for 15 min (Colucci Cante et al., 2022).

The supernatants were collected, and the pellets were re-dissolved in ethanol. This extraction procedure was repeated three times, and the supernatants from all three rounds were pooled together to form the extracts, which were subsequently analyzed for TPC, TFC, and AA. TPC was evaluated using the Folin–Ciocalteu method (Singleton & Rossi, 1965), with some modification (Colucci Cante et al., 2023). In detail, 1 mL of sample extract was mixed with 0.3 mL of Folin–Ciocalteu reagent and 1 mL of $Na_2CO_3$ (7.5% *w/v*), then diluted to 10 mL with deionized water. After 2 h of incubation in the dark at room temperature, absorbance was measured at 720 nm (UV-31 SCAN). Deionized water served as the blank. Total phenolic content was calculated using a calibration curve with gallic acid (0.05–0.25 mg/mL), and results were expressed as milligrams of gallic acid equivalents (GAE).

TFC was determined using the aluminum chloride colorimetric method with slight modifications (Ismail et al., 2017). Briefly, 5 mL of the extract was mixed with 0.5 mL of a 2% (*w/v*) $AlCl_3$ solution, and the volume was adjusted to 10 mL with 70% (*v*/v) ethanol. The mixture was incubated overnight in the dark at room temperature. Absorbance at 420 nm was measured using a spectrophotometer, with 70% (v/v) ethanol as the blank. Total flavonoid content was calculated using a calibration curve prepared with quercetin (0.0015–0.02 mg/mL in ethanol), and results were expressed as milligrams of quercetin equivalents (QE).

AA assay was assessed using the ABTS method with minor modifications (Thaipong et al., 2006). To prepare the working solution (S1), 7.4 mM ABTS and 2.6 mM potassium persulfate (2:1 ratio) were incubated in the dark for 12 h at room temperature. S1 was then diluted with ethanol until an absorbance of 0.7 ± 0.02 at 734 nm (S2) was achieved. For the assay, 0.15 mL of each extract was mixed with 2.85 mL of S2 and incubated for 2 h in the dark. Absorbance was measured at 734 nm. Antioxidant activity was quantified in micromoles of Trolox equivalents (TE), using a calibration curve generated with Trolox standards (25–600 μM).

**Table 1**
Schematic description of the postbiotic samples obtained by free-cell or capsule fermentation, not digested or following digestion.

| Samples | Description |
|---|---|
| FCpb | *Postbiotic obtained by free-cell fermentation* |
| CAPpb | *Postbiotic obtained by capsule fermentation* |
| dFCpb | *Postbiotic obtained by free-cell fermentation post-digestion by INFOGEST protocol* |
| dCAPpb | *Postbiotic obtained by capsule fermentation post-digestion by INFOGEST protocol* |

### 2.5. Characterization of biological activity

#### 2.5.1. Cell culture

The human colorectal adenocarcinoma cell line HCT116 (ATCC CCL-247) was kindly provided by Prof. A. Pollice (Department of Biology, University of Naples Federico II).

HCT116 cells ($2.5 \times 10^5$) were seeded in tissue culture flasks (25 $cm^2$ surface) and grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) at 37 °C and under humidified atmosphere of 5% $CO_2$ in air. The medium was changed twice a week, and cells were sub-cultivated when confluent.

#### 2.5.2. Postbiotic preparation for cell culture experiments

Undigested (CAPpb, FCpb) and digested postbiotic (dCAPpb, dFCpb) were dissolved in PBS to reach a concentration of 5 mg/mL. Finally, samples were centrifuged (15 min, 200 *g*), then the supernatant was collected and used in the experiments described below.

#### 2.5.3. Evaluation of cell survival by MTT cytotoxicity assay

HCT116 cells were seeded into 96-well plates ($1.5 \times 10^4$ cells per well) and cultured for 20 h in complete medium at 37 °C. After removing the medium, cells were rinsed with RPMI, and then incubated (24 h, 37 °C) in RPMI containing different amounts (0.03%, 0.06%, 0.1%, 0.3%, or 1%) of each postbiotic, namely CAPpb, or FCpb, or dCAPpb, or dFCpb. Following incubation, the medium was discarded, and cell viability was assessed using the MTT reduction assay. In detail, 100 μL of MTT (0.5 mg/mL in RPMI without Phenol red) was added to each well, and the plates were incubated for 3 h at 37 °C. Subsequently, 100 μL of a solution containing 0.1 M HCl in isopropanol were then added to each well, and, after 30 min, the absorbance at 595 nm was measured by a Synergy microplate reader. The data were expressed as viability percentage, assuming the absorbance value from untreated cells as 100%.

#### 2.5.4. Evaluation of postbiotics' anti-inflammatory activity

HCT116 cells were seeded into 6 well-plate ($2.5 \times 10^5$ cells per well) in complete medium and cultured for 24 h. After removing the medium, cells were rinsed with RPMI, and incubated (20 h, 37 °C) in RPMI containing 1 μg/mL of LPS, either alone or in the presence of (0.1%) undigested (CAPpb, FCpb) or digested (dCAPpb and dFCpb) postbiotic dry products. At the end of incubation the cells were extensively washed with RPMI, detached by treatment (10 min, 37 °C) with 500 μL of trypsin, and finally lysed with 0.1 mL of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8) supplemented with the protease inhibitor cocktail (1:200, *v*/v) and the phosphatase inhibitor cocktail (1:100, v/v). The cell lysates were centrifuged (14,000 *g*, 20 min, 4 °C), analyzed for their protein

concentration (Bradford, 1976), and used in subsequent experiments.

*2.5.4.1. Western blotting.* Electrophoresis under denaturing and reducing conditions was carried out on 1.5-mm-thick slab gel, using the discontinuous system previously described (Laemmli, 1970), but with a separating gel containing 10% polyacrylamide (Mazzoli et al., 2024). Samples from each cell lysate (15 μg of protein extract) were boiled in the O'Farrell buffer O, containing SDS and β-mercaptoethanol (O'Farrell, 1975), for 5 min before loading on the gel. Protein fractionation was achieved in 90 min at 100 V. Following electrophoresis, proteins were transferred onto nitrocellulose membranes, according to a published procedure (Towbin et al., 1979). After protein blotting, the membranes were rinsed in TBS, left for 15 min in TBS (130 mM NaCl, 20 mM Tris HCl, pH 7.4) containing 0.05% (*v*/v) Tween 20 (T-TBS), and blocked by incubation with 5% BSA (*w/v*) in T-TBS (1 h, 37 °C). After blocking, membranes were incubated overnight at 4 °C with primary antibodies, washed, and then treated (1 h, at 37 °C) with the appropriate peroxidase-conjugated secondary antibodies. Antibody dilutions are listed in Supplementary Table 2. The amount of phosphorylated NFkB, or eIF2α was expressed as relative to the total NFkB, or eIF2α, respectively; accordingly, after revelation of the immunocomplexes, the membranes were submerged in stripping buffer (1% w/v SDS, 25 mM glycine, pH 2; for 30 min, at 37 °C) (Spagnuolo et al., 2018), extensively washed, and then incubated with the specific antibody for the total form of the protein (Supplementary Table 2). For loading control, after detection of each antigen, the membranes were stripped and incubated (overnight, at 4 °C) with mouse anti-β-actin IgG (1:1000 in 0.25% v/v non-fat milk) followed by goat anti-mouse (GAM)-HRP IgG (1:350000 in 0.25% v/v non-fat milk; 1 h, at 37 °C). Signal detection was carried out using the Excellent Chemiluminescent Kit from Elabscience. Densitometric analysis of chemidoc or digital images of X-ray films exposed to immunostained membranes was performed with Un-Scan-It gel software (Silk Scientific, UT, USA).

*2.5.5. Markers of redox homeostasis*

Nitro-tyrosine (N-Tyr) concentration was quantified by ELISA in cell lysate, after incubation with LPS and/or postbiotics obtained as previously described (Cigliano et al., 2019). In detail, cell lysates were diluted (2 ng/μL) with coating buffer (7 mM $Na_2CO_3$, 17 mM $NaHCO_3$, 1.5 mM $NaN_3$, pH 9.6), and aliquots (50 μL) of diluted samples were incubated (overnight, 4 °C) in the wells of a microtiter plate. After four washes with T-TBS and four washes with high-salt TBS (500 mM NaCl, 20 mM Tris-HCl, pH 7.4), the wells were blocked with TBS containing 0.5% BSA (1 h, 37 °C). After washing, the wells were incubated (1 h, 37 °C) with 50 μL of rabbit anti-N-Tyr antibody (1: 700 dilution in T-TBS containing 0.25% BSA) followed by 60 μL of Goat anti-rabbit horseradish peroxidase-conjugated IgG (1:4000 dilution in T-TBS containing 0.25% BSA). Peroxidase-catalyzed color development from *o*-phenylenediamine was measured at 492 nm. Results are reported as OD per mg of total proteins.

For the measurement of Glutathione reductase (GR) activity, after the above reported treatment with LPS in the absence or presence of each postbiotics, cells were detached by scraper, lysed in cold buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA). After centrifugation (10,000 *g*, 15 min, 4 °C), the protein concentration of supernatant was measured (Bradford, 1976), and finally 15 μg of protein extract was used in the activity assay. Glutathione reductase (GR) activity was measured by monitoring the decrease of NADPH absorbance at 340 nm, at 25 °C, using a Glutathione Reductase Assay Kit (Cayman Chemical, USA), according to the manufacturer's instructions. The enzyme activity was calculated using the NADPH molar extinction coefficient, $\varepsilon = 6.22 \times 10^{-5}$, considering that one unit of GR is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADPH per minute. The specific activity was expressed in U/mg of protein.

*2.6. Statistical analysis*

In all experiments, each sample was analyzed in triplicate and, data were expressed as mean values ± standard error (SEM). The statistical analysis program "Graph Pad Prism 9.3.1" (Graph Pad Software, San Diego, CA, USA) was used to perform regression analyses and to compare groups. The comparison of bacterial growth curves, lactic acid production, and glucose consumption were performed using two-way ANOVA followed by Šídák's multiple comparison test, while cell and product Yields and kinetic parameters were compared using the *t*-test. Finally, the comparison of postbiotics in terms of antioxidant and biological activities was performed using one-way ANOVA followed by Tukey's multiple comparison test. A $p$-value $<0.05$ was considered significant in the reported analyses.

## 3. Results

*3.1. Characterization of the fermentation processes: Bacterial growth, lactic acid production, and glucose consumption*

*Lacticaseibacillus paracasei CBA L74* was employed as the fermenting microorganism. Fermentation was conducted using two approaches: a free-cell process, in which the microorganism remained unencapsulated, and an encapsulated process, where cells were immobilized in oat-based alginate capsules. The progress of fermentation was monitored by assessing bacterial growth (CFU/mL), lactic acid production (mg/mL) as the primary metabolite, and glucose consumption (mg/mL), as illustrated in Fig. 1.

Aseptic sampling at $t_0$ and after 2, 4, 6, 16, 18, 20, 22, and 24 h of fermentation allowed the generation of bacterial growth curves (Fig. 1A). In both cases (free-cell and capsule fermentation), the fermentation started at $10^6$ CFU/mL, as intended. Bacterial growth followed a similar trend in both systems until 20 h, after which capsule fermentation supported significantly higher bacterial proliferation compared to the free-cell process (free-cell: $7.05*10^8 \pm 1.95*10^8$ CFU/mL; capsule: $3.58*10^9 \pm 1.42*10^9$ CFU/mL; $p < 0.0001$). The difference was maintained until the end of the process (free-cell: $4.73*10^8 \pm 2.57*10^8$ CFU/mL; capsule: $3.30*10^9 \pm 9.46*10^8$ CFU/mL; $p < 0.0001$). The lactic acid, whose production is shown in (Fig. 1B), was significantly increased for capsule fermentation starting from $t_{16}$ (free-cell: 5.25 ± 0.61 mg/mL; capsule: 15.95 ± 0.49 mg/mL; $p < 0.0001$). By the end of the process, lactic acid concentration in the capsule system was more than twice that observed in the free-cell fermentation (free-cell: 9.08 ± 0.62 mg/mL; capsule: 20.49 ± 1.16 mg/mL; $p < 0.0001$). Looking at the trend of glucose consumption (Fig. 1C), it is possible to note that the microorganism left fermented in capsules needs a higher amount of glucose compared to the one fermented free. However, statistically significant differences were observed only at $t_{16}$ ($p < 0.01$), $t_{22}$ ($p < 0.05$), and at $t_{24}$, where glucose consumption was similar in both processes (free-cell: 7.70 ± 0.30 g/L, with a glucose consumption of 6.47 g/L; capsule: 8.04 ± 1.89 g/L, with a glucose consumption of 4.23 g/L).

*3.2. Characterization of the fermentation processes: Cell and product yields and kinetic parameters*

The two fermentation processes were further characterized by calculating cell and product yields according to Eqs. 1 and 2 described in the Methods section. Capsule fermentation allowed for improved bacterial growth and lactic acid production compared to the glucose consumed (Table 2). Indeed, higher cell and product yields were found for capsule fermentation compared to the free-cell process ($p < 0.05$).

After obtaining the bacterial growth curve for both fermentation processes, key kinetic parameters were calculated using the equations provided. The results are summarized in Table 3. Notably, the fermentation carried out in capsules allowed microorganisms to grow with a higher generation number, although no significant differences were

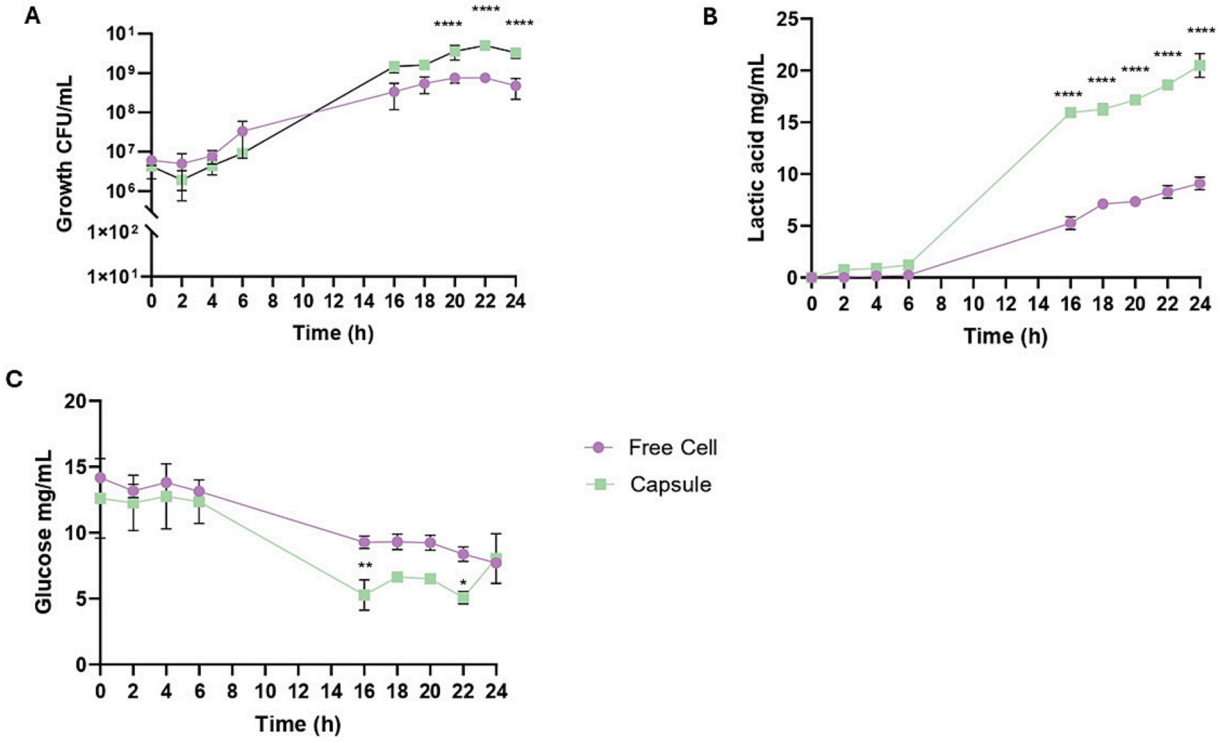


**Fig. 1. Free-cell vs Capsule fermentation**: A) bacterial growth (CFU/mL), B) lactic acid production (mg/mL), and C) glucose consumption (mg/mL). Each result is expressed as the mean ± standard deviation ($n = 3$). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

**Table 2**
Cell and Product Yield for free-cell and capsule fermentation. Each result is expressed as the mean ± standard deviation (n = 3). *p < 0.05.

| | Free-cell Fermentation | Capsule Fermentation |
|---|---|---|
| Cell Yield [mg cells/mg glucose] | 0.12 ± 0.07 | 1.28 ± 0.54* |
| Product Yield [mg lactic acid/mg glucose] | 1.40 ± 0.04 | 4.78 ± 1.73* |

**Table 3**
Kinetic parameters for free-cell and capsule fermentation. Each result is expressed as the mean ± standard deviation (n = 3). **p < 0.01.

| | Free-cell Fermentation | Capsule Fermentation |
|---|---|---|
| Generation number n | 6.63 ± 0.94 | 8.43 ± 1.03 |
| Doubling Time $t_d$ (h) | 2.43 ± 0.32 | 1.43 ± 0.16** |
| Constant Growth rate k ($h^{-1}$) | 0.41 ± 0.06 | 0.70 ± 0.08 ** |
| Specific Growth rate μ ($h^{-1}$) | 0.28 ± 0.04 | 0.49 ± 0.05 ** |

found, as well as higher constant and specific growth rates ($p < 0.01$), and a shorter doubling time ($p < 0.01$) compared to those fermented freely.

### 3.3. Characterization of the Postbiotic dry products

#### 3.3.1. Antioxidant properties

The postbiotic samples, both undigested (FCpb and CAPpb), and following in vitro digestion (dCAPpb and dFCpb), were further analyzed for their TPC, TFC, and AA to assess the impact of the fermentation processes, bacterial load inactivation, and drying on the functional characteristics of the final products. All the results are shown in Fig. 2. It emerged that the digestive process allows an increase of one order of magnitude with statistically significant difference in AA, TPC, and TFC for the postbiotic capsule and free-cell samples (dCAPpb: $p < 0.0001$, and dFCpb: $p < 0.0001$) compared to those not subjected to digestion (CAPpb and FCpb).

#### 3.3.2. Characterization of the biological activity on HCT116 cell model

*3.3.2.1. Effect of oats postbiotics on HCT116 cell viability.* To initially assess the impact of postbiotics on cell viability, HCT116 cells were incubated with different concentrations (0.03, 0.06, 0.1, 0.3, 1%) of each postbiotic, and cell survival was measured using the MTT assay. As shown in Fig. 3, no effect on cell viability was found when cells were incubated with undigested oats postbiotics, while a decrease in cell viability was found when concentrations higher than 0.3% of digested postbiotics were used in cell culture (dFCpb: $p < 0.01$; dCAPpb: $p < 0.0001$). The 0.1% concentration was chosen for further experiments, as described below.

*3.3.2.2. Effect of postbiotics on LPS-activated intestinal cells.* To test the efficacy of each postbiotic, intestinal inflammation was simulated by activating HCT116 cells with LPS. To investigate whether oats postbiotics exert biological anti-inflammatory activity on LPS-induced inflammation, the activation of the pro-inflammatory signaling pathway was evaluated by quantifying the expression of the LPS receptor, namely TLR4 receptor, as well as the MyD88 protein downstream of TLR4, and NFkB phosphorylation (as index of activation of the transcription factor). As expected, LPS treatment led to a significant increase in the protein levels of these inflammatory markers compared to control cells ($p < 0.0001$; Fig. 4A). Simultaneous treatment with LPS and oats postbiotics induced a decrease in level of TLR4, compared to LPS-treated cells, in both undigested and digested samples and both from free-cell and capsule ($p < 0.0001$; Fig. 4A). A significant reduction in MyD88 levels, compared to LPS-treated cells, was obtained in digested samples, both free-cell and capsule (dFCpb and dCAPpb; $p < 0.01$), and in undigested capsule (CAPpb, $p < 0.05$). No significant difference was detected by using the postbiotic produced with free-cell (FCpb)

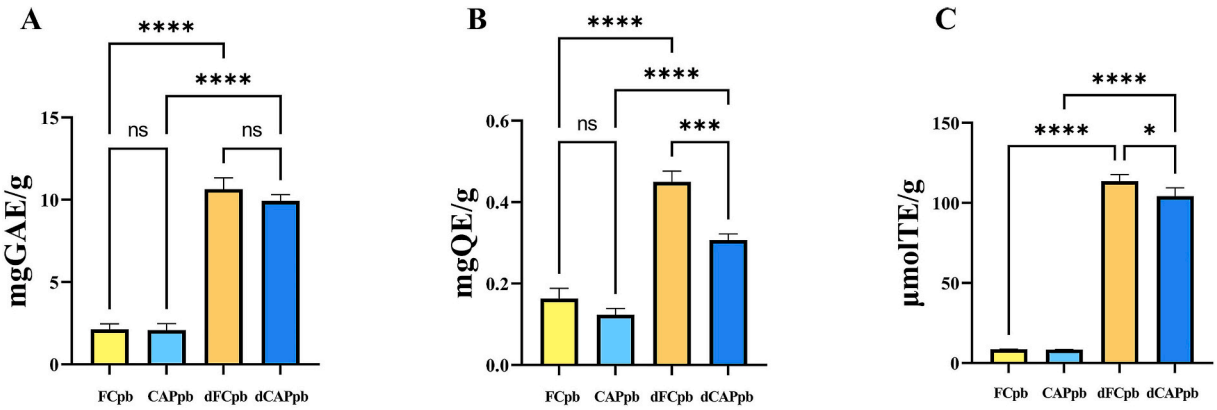


**Fig. 2. Postbiotics characterization.** A) Total Polyphenol Content (TPC), B) Total Flavonoid Content (TFC), and C) Antioxidant Activity (AA) of samples (FCpb, CAPpb, dFCpb and dCAPpb). Each result is expressed as the mean ± standard deviation (n = 3). Statistical analysis compared innovative and traditional fermentation postbiotic * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

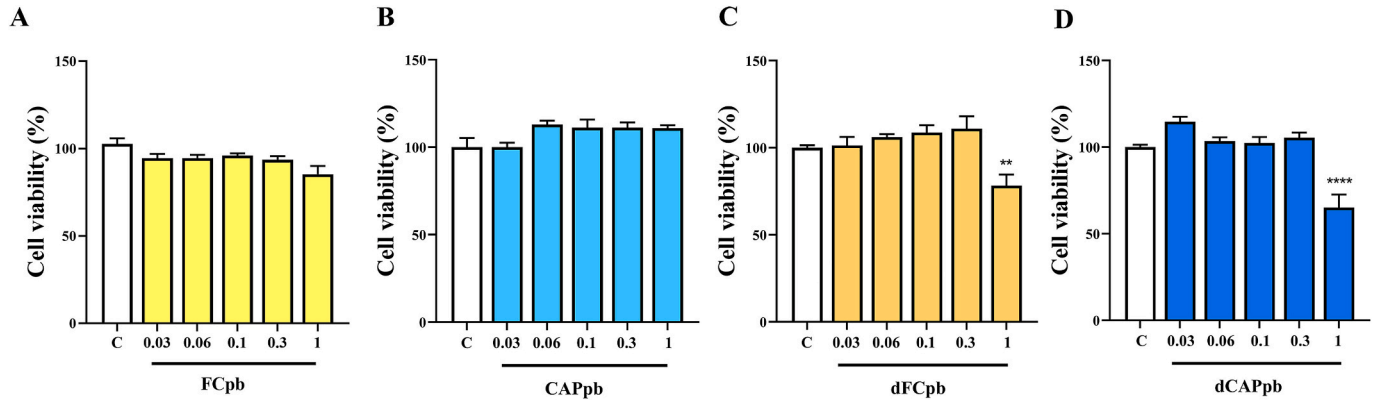


**Fig. 3. Cell viability assay in HCT116 treated with FCpb (A), CAPpb (B), dFCpb (C), dCAPpb (D).** HCT116 cells were incubated into 96-well plate (24 h, $1{,}5 \times 10^4$ cells per well) in RPMI containing 1% penicillin/streptomycin and increasing concentrations of oats postbiotics (0.03, 0.06, 0.1, 0.3, 1%). Cell survival was evaluated by MTT assay, and it was expressed as percentage of viability of the control (cells cultured without postbiotics). Significant differences from control media are indicated. ** $p < 0.01$; **** $p < 0.0001$ compared to C. The data are expressed as mean ± SEM of quadrupled.

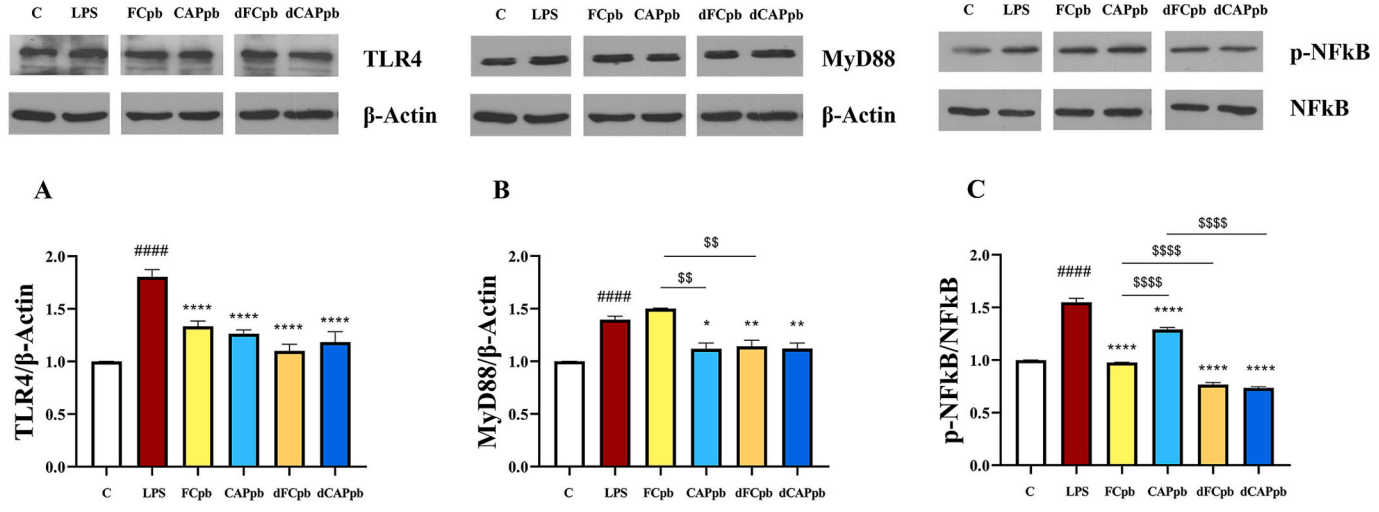


**Fig. 4. Markers of inflammation.** A) Toll-like receptor 4 (TLR4) protein content, B) myeloid differentiation primary response protein (MyD88) protein content, C) phosphorylated NFkB/NFkB ratio (with representative blots, normalized to controls) in HCT116 cells in the absence (C) or presence of LPS (LPS) or LPS plus undigested (FCpb; CAPpb) or digested postbiotics (dFCpb; dCAPpb). The data are expressed as mean ± SEM of three different experiments. #### $p < 0.0001$ compared to C; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared to LPS: $$ $p < 0.01$, $$$$ $p < 0.0001$ (One-way ANOVA followed by Tukey post-test).

(Fig. 4B). Finally, p-NFkB/NFkB ratio was found significantly reduced, with respect to LPS-treated cells, in all samples derived from cells

incubated with each postbiotic ($p < 0.0001$). Interestingly, the effect was more pronounced in the presence of digested samples, both free-cell and capsule (dFCpb and dCAPpb), compared to undigested samples (FCpb and CAPpb $p < 0.0001$; Fig. 4C).

*3.3.2.3. Endoplasmic reticulum stress.* Since LPS-induced inflammation could trigger endoplasmic reticulum stress (ERS), leading to the accumulation of misfolded and unfolded proteins, the modifications of the degree of eukaryotic initiation factor 2α (eIF2α) phosphorylation and the amount of Calnexin, as ERS indicators, were evaluated.

The p-eIF2α/eIF2α ratio, which represents an index of endoplasmic reticulum stress activation, was significantly increased in LPS-treated cells compared to control cells ($p < 0.0001$; Fig. 5A). Interestingly, the p-eIF2α/ eIF2α ratio was decreased, relative to LPS treatment, in cells simultaneously incubated with LPS and undigested postbiotics derived from free-cell (FCpb) or digested postbiotics obtained from free-cell (dFCpb) and capsule (dCAPpb) ($p < 0.0001$; Fig. 5A). However, an increased ratio was observed when incubation with LPS was performed in the presence of undigested capsule ($p < 0.0001$; Fig. 5A). Additionally, Calnexin levels increased in LPS-treated cells, compared to control cells ($p < 0.0001$; Fig. 5B), while a significant reduction relative to LPS treatment was obtained in the presence of both undigested capsule (CAPpb; $p < 0.001$) and digested postbiotics (dFCpb and dCAPpb; $p < 0.0001$). Notably, as shown in Fig. 5B, the effect was more pronounced with digested free-cell postbiotics (dFCpb) compared to undigested free-cell (FCpb; $p < 0.001$), and with undigested capsule (CAPpb) compared to FCpb ($p < 0.05$).

*3.3.2.4. Oxidative stress.* Recent studies have shown that gut inflammation is strongly related to oxidative stress in the enterocyte (Malesza et al., 2021). To investigate whether LPS-induced inflammation is associated with oxidative stress and the potential postbiotics efficacy, nitrotyrosine (N-tyr) levels were evaluated in cell lysates as a marker of nitrosative stress. As shown in Fig. 6A, LPS treatment is associated with an increase in N-tyr ($p < 0.0001$), whereas a significant reduction was observed in lysates from cells treated with both LPS and postbiotics ($p < 0.0001$). Notably, digested capsule was more effective than undigested capsule (dCAPpb vs CAPpb; $p < 0.01$). Furthermore, among the digested postbiotics, the capsule produced a more pronounced decrease of N-tyr than free-cell (dCAPpb vs dFCpb; $p < 0.001$). In response to oxidative stress, one of the main cellular defense mechanisms involves reduced glutathione (GSH) and glutathione reductase (GR) is the enzyme that reduces oxidized glutathione (GSSG) to GSH, thereby restoring redox homeostasis of the cell. As shown in Fig. 6B, a significant reduction of GR activity was observed when cells were incubated with LPS ($p < 0.0001$), while treatment with LPS and postbiotics, both undigested and digested, significantly increased enzyme activity (FCpb, $p < 0.05$; CAPpb, $p < 0.0001$; dFCpb, $p < 0.001$; dCAPpb, $p < 0.0001$). In particular, by comparing undigested samples, capsule was more effective than free-cell (CAPpb vs FCpb, $p < 0.0001$); further, digested free cells produced a higher GR activity increase than undigested ones (dFCpb vs FCpb, $p < 0.01$). Finally digested capsule resulted more effective than undigested (dCAPpb vs CAPpb, $p < 0.05$).

## 4. Discussion

This study demonstrated that oat fermentation employing an innovative process using capsules as mini-bioreactor fermenters was more efficient than the traditional free-cell approach. This was evidenced by enhanced bacterial growth, increased lactic acid production, higher process yields, and improved kinetic parameters.

The higher bacterial growth observed in this study contrasts with previous results, showing no differences in *Lacticaseibacillus rhamnosus GG* bacterial growth between MRS-based calcium alginate gel beads and a free-cell process (Yuan et al., 2023). Conversely, a similar study (Nayedova et al., 2013) found that the yeast immobilized in alginate beads exhibited higher growth efficiency compared to the free-cell. This suggests that the results may be strongly influenced by the type of microorganism, but also by the composition of the capsule.

Lactic acid production in the capsules was more than doubled compared to the free-cell process, reaching values of 22 mg/mL comparable to those previously reported with *Lacticaseibacillus rhamnosus*

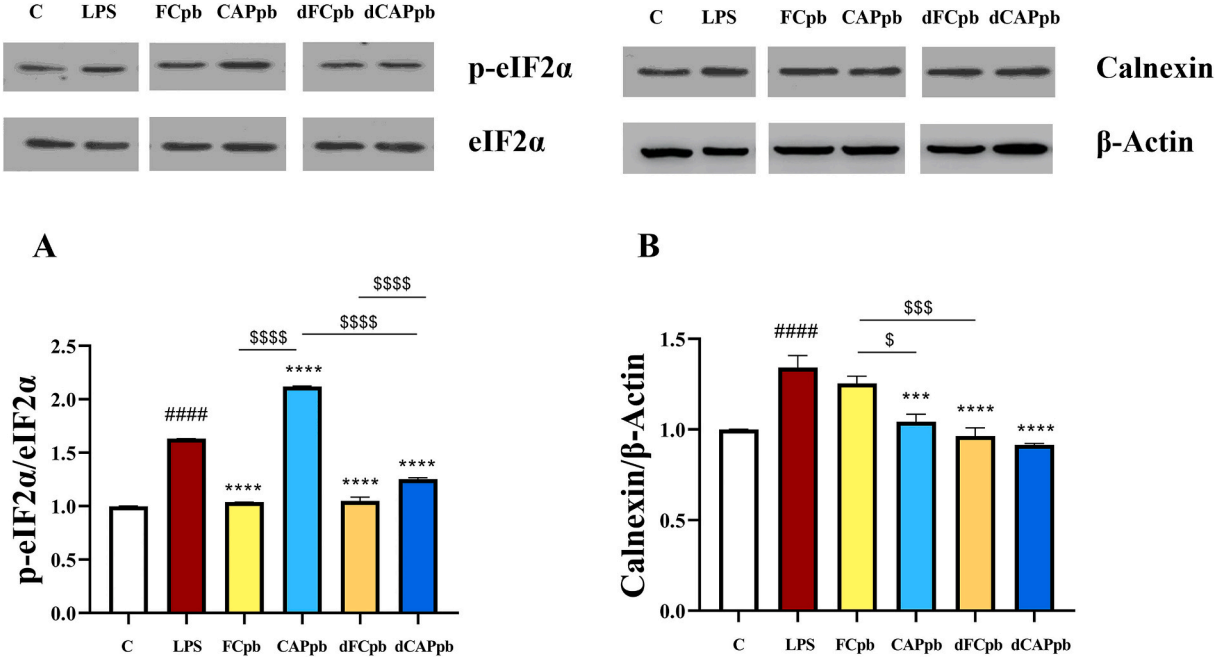


**Fig. 5. Markers of endoplasmic reticulum (ER) stress**. A) phosphorylated p-eIF2α/eIF2α ratio; B) calnexin content (with representative blots, normalized to controls) in HCT116 cells in absence (C) or presence of LPS (LPS) or LPS plus undigested (FCpb; CAPpb) or digested postbiotics (dFCpb; dCAPpb). The data are expressed as mean ± SEM of three different experiments. #### $p < 0.0001$ compared to C; *** $p < 0.001$, **** $p < 0.0001$ compared to LPS: $ $p < 0.05$, $$$ $p < 0.001$, $$$$ $p < 0.0001$ (One-way ANOVA followed by Tukey post-test).

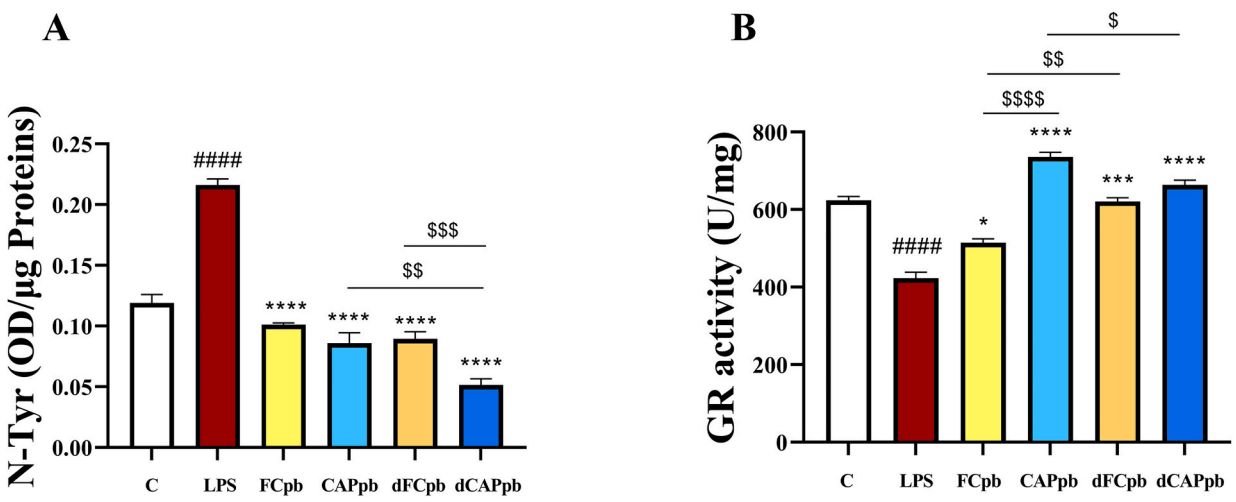


**Fig. 6. Markers of oxidative stress.** Quantification of Nitro-tyrosine (N-Tyr) (titrated by ELISA) (A) and Glutathione reductase activity (B), in HCT116 cells in absence (C) or presence of LPS (LPS) or LPS plus undigested (FCpb; CAPpb) or digested postbiotics (dFCpb; dCAPpb). The data are expressed as mean ± SEM of three different experiments. #### $p < 0.0001$ compared to C; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ compared to LPS: $ $p < 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$, $$$$ $p < 0.0001$ (One-way ANOVA followed by Tukey post-test).

immobilized in alginate pearls (Bahry et al., 2019).

The best fermentation outcomes achieved for the capsule system were further validated by enhanced cell and lactic acid yields. Indeed, the product yield reached 1.40 mg/mg with the free-cell process and, notably, 4.78 mg/mg with capsule fermentation, clearly evidencing the process improvement compared to a previous study, where a product yield value of 0.94 mg/mg glucose was observed for capsule fermentation, compared to 0.96 mg/mg using a free-cells process (Wang et al., 2020).

Capsule fermentation resulted in improved process kinetics, with nearly double the differences observed compared to the free-cell system, including a lower doubling time (td) and higher values of n, k, and µ. Specifically, the doubling time achieved with capsule fermentation was significantly shorter than that reported by another study, which found that *Saccharomyces cerevisiae* encapsulated in calcium alginate exhibited a doubling time of 2.8 h (Galazzo & Bailey, 1990).

In addition, a better duplication capacity of the microorganism during the exponential growth phase was obtained when the fermentation with capsule mini-bioreactors was carried out. The optimization of the process by capsule fermentation may be due to the reduction of the reaction volumes, with consequent reduction of spatial inhomogeneities. The scale-down of the process, obtained by the use of capsule mini-bioreactors, allows the creation of both an optimal microenvironment and an efficient transport of nutrients and metabolites that improves the enzymatic activity of microorganisms, as well as the production of lactic acid. It can be hypothesized that process optimization may rely on activating alternative metabolic pathways to glucose, such as those involving alanine and other amino acids, which can be converted into pyruvate as a reaction intermediate, as suggested by previous research (Fernández & Zúñiga, 2006). This shift could result in reduced overall glucose consumption compared to the free-cell system, while significantly increasing yields in the innovative system. Moreover, cell immobilization enables the establishment of a high-cell-density process, which, on one hand, serves as an effective strategy to maximize lactic acid production (Abdel-Rahman et al., 2013), but on the other hand, may induce cellular stress that promotes the activation of alternative metabolic pathways. This phenomenon may also explain the comparable glucose consumption between free-cell and capsule processes (Li et al., 2023), the latter with a significantly higher lactic acid production. Notably, the postbiotic samples subjected to the digestive process (dFCpb and dCAPpb) showed a higher content of polyphenols, flavonoids, and considerably improved antioxidant activity compared to the samples not subjected to digestion (FCpb and CAPpb). These results are in line with a previous study showing that plant-based burgers exhibited enhanced antioxidant capacity after undergoing digestion, most likely due to the activity of digestive enzymes that facilitate the release of antioxidant compounds (Kowalczewski et al., 2024).

Of interest, a postbiotic product of oat gruel fermented with *L. plantarum 299v* was shown to exert a beneficial effect on the intestinal epithelial barrier function of patients with irritable bowel syndrome, underscoring the clinical potential of oat-based postbiotics in treating intestinal dysfunction (Bednarska et al., 2022). This finding supports the choice of oat as an optimal substrate for developing novel fermentation processes to produce postbiotics with beneficial biological properties. Since inflammation is a central feature of various intestinal diseases, including Inflammatory Bowel Disease (IBD) (Sakurai & Saruta, 2023), the potential anti-inflammatory activity of the oat postbiotics samples from *Lacticaseibacillus paracasei* designed in this study was tested. Indeed, although different studies demonstrated the efficacy of postbiotics in managing intestinal and metabolic disorders (Abbasi et al., 2022; Gasmi et al., 2022), the underlying mechanisms remain largely unexplored. In this work, oat postbiotic samples were shown to reduce the inflammatory pathway induction in intestinal cells activated with LPS. Following LPS binding to its receptor TLR4, adaptors such as MyD88, a downstream protein of TLR4 activation, initiate a signaling cascade leading to NFkB activation, which trigger the expression of genes involved in inflammation (Kawasaki & Kawai, 2014; Wei et al., 2023). Specifically, this study demonstrated that the postbiotics were effective in reducing LPS-induced activation of NFkB pathway, as a significant decrease of both TLR4 and MyD88 as well as of NFkB activation, was found in cells concomitantly treated with LPS and the postbiotics compared to cells activated with LPS. Mechanistically, these results support prior reports demonstrating the beneficial effect of oat-based postbiotics on the colonic mucosal barrier of IBD patients (Bednarska et al., 2022), on transepithelial resistance of Caco-2 cells (Bednarska et al., 2022), and on the repair of rat intestinal barrier damaged by excessive sucrose consumption (Song et al., 2024). Particularly intriguing is the higher anti-inflammatory activity exerted by digested postbiotics, both from free-cells and capsule fermentation. It can be speculated that fermentation followed by digestion led to the release of bioactive compounds able to interfere with the exposure and activation of TLR4 onto the membrane, thus preventing the activation of the NFkB signaling pathway. Supporting this hypothesis, certain polyphenols have been shown to prevent TLR4-mediated inflammation by

blocking LPS-TLR4 binding and downregulating TLR4 expression via microRNA modulation (Coutinho-Wolino et al., 2022).

Inflammation can also trigger ERS (Cao et al., 2016; Li et al., 2020), which in turn activates downstream signaling pathways leading to NFkB activation, with consequent amplification of the inflammatory response (Chipurupalli et al., 2021). The present data demonstrate that LPS activates ER stress in intestinal cells, as demonstrated by increased levels of calnexin and the peIF2α/eIF2α ratio. Interestingly, this study provides the first evidence that oat postbiotics, by preventing LPS-induced inflammation, were also effective in reducing LPS-induced ER stress and restoring protein homeostasis. This effect aligns with recent reports describing the efficacy of *P. acidilactici* GKA4 postbiotics in modulating ER stress in renal tissue (Lin et al., 2024). Consistent with the oat postbiotics beneficial impact on inflammation, the counteracting effect against ER stress was again more pronounced with digested postbiotics. Consistently, reduced oxidative stress was measured in cells concomitantly treated with LPS plus undigested or digested postbiotics. In this context, it should be mentioned that HCT116 cells, when incubated with LPS, exhibit oxidative stress, characterized by high levels of superoxide radical and nitric oxide (Franceschelli et al., 2019). When overproduced, nitric oxide reacts with oxygen radicals forming peroxynitrite, a strong oxidizing and nitrous agents, resulting in nitrosylation of cellular proteins, an irreversible form of protein damage, that can be involved in the pathogenesis of several diseases, including intestinal disease (Potoka et al., 2002; Wang et al., 2021). Given the oat content of polyphenols and flavonoids, the oat postbiotics were able to reduce oxidative stress as evaluated by measuring N-Tyr content, in line with previous findings showing the antioxidant effects of *Lacticaseibacillus casei*-fermented sprouted oat extracts (Cho et al., 2024). In particular, all postbiotics reduced the N-tyr levels compared to cells treated with LPS, with digested capsule-derived postbiotics showing a greater reduction than those from free-cell fermentation. A similar trend, though less pronounced, was observed between undigested CAPpb and FCpb. Moreover, as the digested product dCAPpb showed a better efficacy in reducing N-Tyr compared to CAPpb, it can be postulated that the use of a capsule as a fermentation strategy contributes to promoting the production and/or preserving antioxidant metabolites. This also suggests that oat postbiotic activity of counteracting oxidative stress might be enhanced by the action of digestive enzymes, which allow the release of antioxidant metabolites (Kowalczewski et al., 2024), in line with the higher content of polyphenols, flavonoids, and antioxidant activity found in digested samples compared to not digested samples. Additionally, all four postbiotics were able to trigger the defensive antioxidant enzyme GR, since the LPS-induced decrease in its activity was prevented by the treatment with each postbiotic. This observation, reported here for the first time in activated intestinal cells, is consistent with previous results in *Mytilus galloprovincialis* fed diets enriched with kefir-derived postbiotics (Maione et al., 2025). A significant higher effect was found with undigested postbiotics derived from capsule fermentation CAPpb compared to those derived from free cell fermentation FCpb.

Importantly, the concentrations of postbiotics used in this study did not compromise cell viability, suggesting that their beneficial effects are not associated with cytotoxicity. Since the innovative fermentation process in capsule is carried out using a matrix (oat), a capsule component (alginate), and a microorganism (*L. paracasei*), all of which widely used both in vitro and in vivo studies, it is reasonable to hypothesize that postbiotic capsules represent a safe functional food. The observed anti-inflammatory and protective effects further support their potential future use in humans. This hypothesis is reinforced by evidence regarding the safety, bioactivity, and pharmaceutical properties of postbiotics (Da et al., 2024; Homayouni Rad et al., 2020). The safety of postbiotic capsules in human nutrition will be confirmed by future studies.

## 5. Conclusion

Overall, this study demonstrates that fermentation within the capsules led to increased bacterial growth and lactic acid production, with improved yields and kinetic parameters compared to traditional free-cell fermentation. To the best of our knowledge, no other research has examined the use of an oat-based postbiotic in this manner, evaluating the biological capacity of encapsulated oat-based postbiotics rather than dried and digested oat-based postbiotics. Specifically, both undigested and digested postbiotics (from free-cell and capsule fermentation) showed beneficial anti-inflammatory and antioxidant activity in LPS-activated HCT116 cells, suggesting their potential to reduce intestinal inflammation and applications in nutraceuticals, foods, and cosmetics.

## CRediT authorship contribution statement

**Giulia Lentini:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesca Passannanti:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesca De Palma:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Marianna Gallo:** Validation, Supervision, Data curation, Conceptualization. **Natasha Petecca:** Writing – review & editing, Investigation, Formal analysis. **Maria Stefania Spagnuolo:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Andrea Budelli:** Supervision, Conceptualization. **Roberto Nigro:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Luisa Cigliano:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Data curation.

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## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.148829.

## Data availability

Data will be made available on request.

## References


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