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Microencapsulation of microbial antioxidants from *Mucor circinelloides*, their physico-chemical characterization, in vitro digestion and releasing behaviors in food

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Abstract

This study aimed at increasing the stability of heat-labile and pH-sensitive microbial antioxidants by the microencapsulation. Microbial antioxidants from *Mucor circinelloides* were microencapsulated. The physico-chemical and powder flowing properties of resulting microcapsules were evaluated. The initial safety studies were evaluated by in vivo acute oral toxicity tests. The bio-accessibility of powders vs. extracts was analyzed in in vitro digestion models with further application of microcapsules to model food system. Physico-chemical properties were significantly different ($p < 0.0001$) for all microcapsules regardless of their non-substantial variations ($p > 0.05$) in powder flowing properties. The microencapsulation of extract with 5% whey protein hydrogels (WPHG) + 5% pectin (T_A) showed higher retain-ability of polyphenols accompanying low degradation in gastric and intestinal digestion and with no major toxicity signs. The addition of T_A microcapsule did not produce any nutritional, physico-chemical, compositional, and nutritional distinctions in cheese. Microencapsulation proved to be appropriate approach for not only protecting the thermo-labile and pH-sensitive microbial antioxidants but also for enhanced bioavailability, and targeted release of bioactive extracts.

Keywords: Microbial antioxidants, Microencapsulation, Freeze drying, Cottage cheese, *Mucor circinelloides*, Release behavior

Introduction

The concept of "microbial antioxidants" was first time presented in literature by our previous works in which microbial production of antioxidants was carried out by exploiting the three important oleaginous strains of filamentous fungi called as *Mucor circinelloides* (MC) [22, 23]. These three important strains were MC CBS 277.49 (MCCBS277.49), MC CBS 108.48 (MCCBS108.48), and MC WJ11 (MCWJ11). This work exceptionally declared the candidacy of two strains of MC (i.e., MCCBS 277.49 and MCWJ11) for the microbial production of antioxidants with their possible applications in food and pharmaceutical industry. MC is considered a true "microbial factory" due to its inherent capability of microbial production of organic acids, enzymes, lipids, ethanol, food colorants (β-carotene and lycopene), essential amino acids, chitin, chitosan, and polyphenolic antioxidants [22, 23]. Polyphenols were the major antioxidant components in the mycelia extracts of MC [23]. The characterization of these polyphenolic rich extracts of identified polyphenolic compounds majorly belonging to lipid amides,

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alkaloids, pigments, benzoic acids, terpenoids, cinnamic acid, benzopyrans etc. classes of polyphenolic compounds [22].

The storage and stability studies under accelerated processing conditions and biological buffers mimicking the human fluids revealed the pH-sensitivity, and thermo-labile nature of these polyphenolic antioxidants [22]. The degradation of these microbial antioxidant compounds may hinder the potential effectiveness of these microbial antioxidants in food/nutraceutical and pharmaceutical applications. To overcome these limitations and to increase the stability of antioxidants, and protect their (bio)functionalities, microencapsulation has been exploited as a trustworthy technique to avert the undesirable annihilation of bioactive compounds. The microencapsulation of microbial secondary metabolites may not only preserve and stabilize the antioxidants but also can improve the retention time of antioxidants with improved bio-digestibility/bio-accessibility, offer controlled and targeted release, hinder the adverse interactions among food matrices and bioactive compounds, and masking the astringent taste and smell of bioactive compounds [3].

Now a days, various sophisticated (bio-functional) wall materials and microencapsulation techniques have been developed. The microcapsules produced by each method are different [17]. From the available microencapsulation techniques, the freeze-drying approach was selected for microencapsulation, despite of its high input cost, to further limit the possibilities of antioxidant degradation. Furthermore, the material for encapsulation possesses equal significance like the way encapsulation executed. Among the numerous, commonly employed wall materials for encapsulating the polyphenols rich extracts, pectin, partially hydrolyzed guar gum (PHGG), and whey protein hydrogels (WPHG) are one of them. The plant based polysaccharide known as pectin possess robust film forming, binding, and effective emulsion stabilizer properties. Pectin is also resistant to digestive enzyme, and, hence, used to attain the exact spot of specific delivery of bioactive compounds due to its pH-dependent delivery system and pH-sensitive behavior [53]. Moreover, the approach of controlled/partial enzymatic hydrolysis of guar gum is used to produce PHGG. PHGG is less viscous, low molecular weight, stable, odorless, tasteless, low water holding capable biopolymer with varying solubility features across the wide range of pH [28]. PHGG is least studied functional biopolymer as a wall material but it does provide additional health gains with respect to minimum bio-accessibility of fat and cholesterol in gut [36]. WPHG is heat tempted whey protein gel with preferred pH-predisposition, oriented permeability, and mechanical characteristics. WPHG is another potential good biodegradable carrier of bioactive compounds for site-specific and controlled release of bioactive compounds without any further chemical/enzymatic treatment [21]. However, despite the good and dynamic wall features, an encapsulating material hardly offers all the desires encapsulating characteristics. Thus, the use of mixture of two encapsulating biopolymers is the ultimate way to further widen the efficiency of encapsulation.

Following microencapsulation, appraisal of stability of resulting microcapsule powders during in vitro gastrointestinal conditions and in oil-in-water emulsion system (i.e. cottage cheese) was conducted and compared with non-microencapsulated extracts. Simulated in vitro digestion model was selected for control release studies as these kinds of models are cheap, easy to perform without the need of any high-tech equipment. Additionally, in simulated in vitro digestion models, bio-accessibility of lipophilic or hydrophilic phytochemicals can be assessed by simple centrifugation [35] centrifugation/filtration (through 0.2 μm or even 0.02 μm filters) [29], or, alternatively, following diffusion through semipermeable membranes (dialysis), with e.g. 10,000–12,000 Da cut-offs [8]. For application of powders, oil-in-water (o/w) emulsion system selected. Various food products represent the oil-in-water (o/w) emulsion systems i.e. mayonnaise, (coconut)yoghurt, butter, cheese etc. However, cottage cheese was used, as most of studies in literature described the effect of antioxidants in simple o/w system whereas studies dealing with real o/w systems are rare [26]. In short, the objective of current report was to microencapsulate microbial antioxidant rich extract from MCWJ11 and studied the physico-chemical, powder-flowing, in vitro bio-digestibility/bio-accessibility of consequent microcapsules. Last but not least, toxicity, palatability, nutritional and chromatic appraisal of the cottage cheese with added free and microencapsulated powder was also executed along consumer liking data.

Materials and methods

Chemicals, solvents and other reagents

All the (bio)chemicals materials, solvents or reagents utilized in this work were of biological/spectroscopic/analytical grade or otherwise higher where appropriate. Anhydrous, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), anhydrous monobasic potassium phosphate, Foline Ciocalteu (FC) reagent, aluminum chloride, gallic acid, sodium nitrite, sodium hydroxide, sodium carbonate anhydrous, anhydrous ascorbic acid, trichloroacetic acid, ferric chloride, sulfuric acid, potassium-hexacyanoferrate, and catechin were procured from Sigma-Aldrich GmbH (Sternheim, Germany) whereas analytical-grade methanol, acetonitrile ethanol, potassium sorbate (E202), and acetone were provided by Sigma-Aldrich (St. Louis,

MO, USA). Ciocalteu reagent, pancreatin, 2, 20-azobis-amidinopropane (ABAP), pepsin and dichlorofluorescein diacetate (DCFH-DA) were attained from Sigma (St. Louis, U.S.). Highly-pure water (18 MΩ/cm) was attained from Milli-Q purification device (Millipore Co. USA). Sodium hydrogen carbonate was purchased from Merck (Germany). Sea sand with the grain size of 200–300 was supplied by Scharlau (Barcelona, Spain). Micro-encapsulating material used was whey protein hydrogels (WPHG), pectin, and partially hydrolyzed guar gum (PHGG) supplied by a local manufacturing company (Guangzhou Zio Chemical Company Ltd., PR China).

Strain and culture conditions

The filamentous fungi *M. circinelloides* strain WJ11 (MCWJ11) isolated from soil at Jiangnan University (Wuxi, Jiangsu, PR China) and kindly provided by Dr. Xin Tang, State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, PR China, was grown on solid agar Kendrick and Ratledge (K & R) media for 5 days at 28 ± 2 °C to harvest spores. Aliquots of 100 µL of freshly harvested spore suspensions (10^7 spores/mL) of high lipid producing filamentous fungus MCWJ11 were measured spectrophotometrically and inoculated into liquid K and R medium (150 mL) held in 1 L flasks having baffles (New Brunswick Scientific, Co., Ltd., Edison, NJ, USA) to improve aeration [23] and incubated overnight at 30 °C with 150 rpm shaking. The K and R medium contained 3.3 g/L diammonium tartrate, 30 g/L glucose, 2.0 g/L Na_2HPO_4, 7.0 g/L KH_2PO_4, 1.5 g/L yeast extract (*Saccharomyces cerevisiae*), 1.5 g/L $MgSO_4{\cdot}7H_2O$, 8 mg/L $FeC1_3{\cdot}6H_2O$, 0.1 mg/L $CaC1_2{\cdot}2H_2O$, 0.1 mg/L $CuSO_4{\cdot}5H_2O$, 1 mg/L $ZnSO_4{\cdot}7H_2O$, 0.1 mg/L $MnSO_4{\cdot}5H_2O$ and 0.1 mg/L $CO(NO_3){\cdot}6H_2O$, pH 6.0. Fermenters (2 L; BioFlo/CelliGen-310/115, New Brunswick Scientific, Co.) containing 1.5 L modified K and R medium (2 g diammonium tartrate, 80 g glucose/L plus the inorganic salts, as listed above, were used to incubate 10% (v/v) overnight cultures for inoculation for 120 h in the 2 L fermenters. The modified K and R medium has 2.5 folds higher glucose with 90% reduction in N2 and P. The pH was maintained at 6.0 by auto-addition of 2 M H_2SO_4 or 4 M KOH [23, 24, 30].

Preparation of extracts by pressurized liquid extraction (PLEx)

After 5 days of fermentation using the physiological stress medium, the mycelia were collected using filtration through single layer of muslin cloth, washed thrice with Milli-Q water (Milli-Q Ultrapure Lab-water System, Merck Millipore, Co., Darmstadt, Germany), lyophilized (6 L FreeZone Freeze Dryer, Labconco Corp., Ltd., Kansas City, MO, USA) and pulverized (HC-350 Damai grinder, Yongkang Tianqi Shengshi Industry and Trade Co., Pvt., Ltd., Hangzhou, Zhejiang, PR China) in the presence of liquid nitrogen until finely powdered and screened (1.0 mm). The pressurized liquid extraction (PLEx) was done in a Dionex ASE 350 system (Dionex, Sunnyvale, CA, USA) from the initial lyophilized mycelia. Aliquots of 5 g of lyophilized filamentous fungi were mixed with diatomaceous earth (1:1) (Qingdao Tuosheng Diatomite Co. Ltd., Qingdao, Shandong, PR China) and placed in 34 mL stainless steel cells (Dionex™ ASE™ 150/350, Dionex). The extraction was done in triplicate with absolute ethanol with the following conditions: t = 15 min extraction time, 10.3 MPa at 70 °C. Organic solvent was evaporated at 40 °C in a rotary vacuum evaporator, while the remaining moisture/water was removed using lyophilization [24]. During solvent evaporization, the extracts were kept under nitrogen 20–30 min and kept in screw-capped amber-colored glass bottles at − 80 °C until analyzed in duplicate, for a maximum of 12 weeks.

Microencapsulation powders preparation

In order to formulate the specific dispersions, lyophilized microbial extracts (1.5 g) were diluted with (Milli-Q) water (100 mL) (Milli-Q Ultrapure Lab-water System, Merck Millipore, Co., Darmstadt, Germany) and these "1.5% (w/v) aqueous extracts" were further mixed (GS585-worktop multifunctional vacuum blender, Shanghai Guosheng Industrial Co., Ltd., Shanghai, PR, China) independently with pre-selected wall materials (10% w/v) as follow: 5% WPHG + 5% pectin (w/w) (henceforth denoted and discussed as TA), 10% WPHG (henceforth denoted and discussed as T_B), (5% PHGG and 5% pectin) (w/w) (henceforth denoted and discussed as T_C), and (10% of PHGG) (henceforth denoted and discussed as T_D). The above four sets of dispersions (10 g each) were rotated at 7500 rpm for 7 min using an Ultra-Turrax (T25, IKA) individually and successively lyophilized using Alpha 1–4 LD plus freeze-drying unit (Labconco, Germany). For freeze-drying, the four dispersions initially freeze at − 80 °C for 24 h and followed by drying in the above-mentioned freeze-drying unit at − 70 °C at vacuum pressure less than 25 µmHg for 96 h. After this, the microencapsulated material was crumpled with mortar and pestle. A fine and uniform crumpled powder was collected by using a mesh of size ≤ 8 mm. This uniformed size crumpled microencapsulated powder from four treatments were instantly placed in dark colored polyethylene bags and placed in aluminum sacks. The aluminum pounches were deposited in a desiccator comprising silica for additional analyses, which were executed in triplicate.

Physico-chemical properties of microencapsulated powders

Moisture contents were determined according to the method of AOAC [4] (No. 943.06, section 37.1.10B). Briefly, following heating at 105 °C in drying oven, weight loss of samples was noted. Water activity (A_w) was calculated by undeviating readings in an electronic meter (Aqualab Dew Point 4TEV) at 25 °C for 15 min, after samples were stabilized. Solubility of microencapsulated powders was calculated conferring to method adopted from Cano-Chauca et al. [10], with some variations. Aliquot 1 g of powder from each treatment was mixed with 100 mL of deionized and distilled water tailed by stirring in a magnetic stirrer for at least 40 min. Following, the solution was centrifuged at 9000 rpm (Centrifuge 5810/5810R, Eppendorf AG, Hamburg, Germany) for 7 min. Carefully, the supernatant (25 mL) was transferred to Petri dish of known weight and oven-dried instantly at 105 °C for 4–5 h. The solubility was measured by difference of weight and expressed in percentage (%). Hygroscopicity of powders was measured rendering to the technique dictated from Cai and Corke [9]. Briefly, aliquot 1 g of microcapsule powder was dried at 25 °C with NaCl saturated solution (75.3%). After 7 days, the hygroscopicity was articulated in percentage (%) of adsorbed moisture by weighing the samples.

Differential scanning calorimetry (DSC) (DSC Q2000, TA Instruments, New Castle, DE) was employed to calculate the Glass transition temperature (Tg) of microcapsules. Aliquot 8 mg of sample in aluminum was placed enclosed pans (Tzero, TA Instruments), whereas empty Al (aluminum) pan was taken as standard. The ultra-pure nitrogen was purge gas (flow 45–50 mL/min) with 70 °C to 120 °C temperature range at a heating rate of 30 °C/min. The Tg values were measured using the TA Universal Analysis version 5.5 software. Morphology and particle size of uniform sized crumpled microencapsulated powder manufactured with various wall materials was examined in scanning electron microscope (SEM) (QUANTA-250, FEG Co., Holland), conferring to method of Shui and Leong [45]. There are many methods to determine the size and size distribution i.e. dynamic light scattering (DLS), microscopy, SEM etc. SEM uses a high energy beam of electrons (20–30 keV) to create an image with higher magnification (>100,000×) and resolution capabilities (down to 10 nm) than optical microscopy. The technique has seen widespread use in size analysis of microcapsules [7]. Therefore, the surface morphology and particle size distribution pattern of microcapsules was observed by SEM. Powder particles were attached to an aluminum stub with double-sided sticky tape. The specimens were sputter coated with gold in an ionic sputter coater (SCD-005, BAL-TEC Co., England). They were viewed and photographed in the SEM at an accelerating voltage of 10 kV. Examinations were made at 500× magnifications. The particle size distribution and average particle size examination were accomplished using ImageJ (NIH, Bethesda, MD), where 50–200 microparticles were calculated discretely from the micrographs of SEM. The average particle size/diameter/span was described in μm.

Flow properties of microcapsules

Bulk density, percentage compressibility, angle of repose, particle density, and tapped density were calculated. Toluene was used as a reference to measure the particle density by a pycnometric method. The measured particle density defined as the total mass of particle per its total volume. Graduated glass cylinder (25 mL) was used to determine the Bulk density of ten grams of loosely weighed powder. The initial/original volume (V_0) was noted and which divided the sample weight to calculate bulk density. The "Tappin" approach was used to determine the compact density [15]. The probe is smash on the plane surface area to volume constant. The weight of the sample is divided by the final volume (V_f) taken by the sample to measure compact density. The ration of compression is employed to measure the percentage compressibility and the Hausner ratio is measured with the relation

$$Hr = V0/Vf.$$

For the angle of repose test, 5 g of powder was used to determine angle of repose which was added to a flat surface via a dropping funnel located at 10 cm of that flat surface, impeding the withdrawal there from. Afterwards, the height (h) and the radius (r) of the cone formed was determined by the tangent of the angle given by h/r ratio [20]. The microencapsulation yield was determined based on the preservation of the total phenolic contents (TPC) after the microencapsulation [39]. For this, TPC of raw extracts used for microencapsulation and TPC of microcapsules-drawn-extracts were measured according to method described below. For calculating the TPC of microcapsules-drawn-extracts, microcapsules were extracted using the method of Cilek et al. [12] with some adjustments. Concisely, an aliquote sample (100 mg) of each powder was disseminated for 3 min in ethanol (5 mL) with subsequent filtration using microfilter (0.45 μm) and TPC was recorded by spectrophotometer (Shimadzu Corp., Ltd., Kyoto). The equation for calculating the microencapsulation yield is as follows

$$\begin{aligned}&\text{Microencapsulation yield (\%)}\\&\quad = [1 - \text{TPC of microcapsules-drawn-extracts/}\\&\qquad \text{TPC of raw extracts}] * 100.\end{aligned}$$

Determination of bioactive compounds and their bioactivities after microencapsulation

The three important microbial antioxidant components measured were total phenolic content (TPC), total flavonoid content (TFC) and total condensed tannins (TCT). The TPC of mycelium extracts (5 mg/mL) was measured by adopting a spectrophotometric (UV-1800 Spectrophotometer, Shimadzu Corp., Kyoto, Japan) method as described in Julkunen-tiitto [27] and Slinkard and Singletod [47]. Briefly, sample extracts (250 µL) were mixed with 250 µL of 10% (v/v) FC reagent, followed by the addition of 500 µL saturated sodium carbonate (10%, w/v aqueous solution). After 2 min of incubation at room temperature (20 ± 5 °C), the mixture was placed in the dark for 1 h. Absorbance was then measured at 750 nm. Gallic acid was used to obtain the calibration curve (10 to 100 µg/mL), and the concentration of TPC was expressed as gallic acid equivalent (GAE)/g of dry extract.

TFC was measured spectrophotometrically using the $AlCl_3$ method [48]. Briefly, each extract (0.5 mL) of 10 mg/mL was mixed with 1.0 mL of 2% (v/v) $AlCl_3{\cdot}6H_20$ absolute ethanolic solution and the absorbance was measured after 10 min at 430 nm. Quercetin was used to obtain the calibration curve (0.01–50 µg/mL), and the concentration of TFC was expressed as quercetin equivalent (QE)/g of dry extract.

Similarly, TCT was determined using a previously developed method by Julkunen-tiitto [27] and Slinkard and Singletod [47] using catechin as a reference compound to obtain a standard curve (50–1000 µg/mL). Briefly, an aliquot of each extract (50 µL) was mixed with 1.5 mL of 4% (w/v) vanillin in absolute ethanol, followed by the addition of 750 µL concentrated HCl. The mixture was allowed to stand at room temperature for 20 min in the dark. The blank was 4% (v/v) concentrated HCl in absolute ethanol. The absorbance was read at 500 nm against a blank. Results were expressed in catechin equivalent (CE)/g of dry extract which reflects the tannin content.

The sustainability of antioxidative activities of same consequential microencapsulated powders were appraised in terms of total antioxidant activity evaluated by β-carotene bleaching assay (TOAA), cupric ion reducing antioxidant capacity (CUPRAC), and chelating effects on ferrous ions assays (CHELAT). All these spectrophotometrically analysis were accomplished conferring to our formerly developed procedures for MC [22, 23]. The chelating activity was articulated as EC_{50}, the concentration (in mg/mL) that chelated 50% of the Fe^{2+} ions. So the outcomes of CHELAT were standardized and articulated as EC_{50} values (mg extract per mL) for appraisal. Effectiveness of antioxidant properties is inversely correlated with EC_{50} value.

Release kinetics of microencapsulated powder during simulated in vitro enzymatic digestion

Simulated enzymatic digestion was carried out in line with the previously reported literature procedure with slight modifications [38]. Since ethanolic extracts of MCWJ11 were found to be rich in phenolic compounds [22, 23] so the release kinetics of phenolic compounds was also focused in this in vitro digestion model. Gastrointestinal (GI) digestion is of two phases: gastric and intestinal digestion. Pure ethanolic extracts and mixed microcapsule-mycelial solution (henceforth referred and denoted as matrix solution) were examined as standard samples. The incubation of samples was done in (simulated)gastric fluid condition devoiding enzyme (SGDig, pH 1.2) and fed-state condition having simulated intestinal fluid containing pancreatin (FSIDig, pH 6.8) at 37 ± 1 °C. To ensure the effective diffusive release of phenolics in the aqueous medium and for the appraisal of acidic pH impact on stability of TPCs, as compared to stability of TPCs in FSIDig, pepsin was not added in SGDig.

Release media

The gastric conditions were created devoiding any enzyme denoted as SGDig pH 1.2 (USP 32) while (small) intestinal condition (FSIDig) was phosphate buffer pH 6.8 (46.6% disodium hydrogen phosphate dihydrate 1/15 M, 53.4% potassium dihydrogen phosphate 1/15 M) accompanied with 15 mM bile salts, 3.75 mM phospholipids, 150 mM sodium chloride, 450 U/mL pancreatin as enzyme source, and 5 mM calcium chloride [38]. The bile acid content of the heterogenic porcine bile extract used was assayed using the enzymatic Ecoline S+ test (Diagnostic Systems, Holzheim, Germany) and adjusted as appropriate prior to its addition.

In-vitro release systems

In vitro release kinetics of microcapsule powder and degradation kinetics of nonencapsulated polyphenol rich ethanol extracts were monitored during continuously agitated (10 rpm) and incubated vessel, agitated about parallel hatchets, in an end-over-end agitator furnished with a control system of temperature. Prior to start the experiment release media and capsule powder were warmed to 37 ± 1 °C, and the whole system was maintained at that temperature in course of each incubation. Samples of both microbial ethanolic extracts (nonencapsulated and undigested) and microcapsule-loaded extracts (undigested) were impregnated (at rate so that initial TPCs/polyphenolics concentration was set to 0.7%) to the incubation media. This concentration was recognized to be referenced as 100% (around 6000 GAE)/kg of dry extract), and all successive concentrations of

succeeding samples were taken in relation to this value. Ensuing standard pharmacopoeia approach (Ph. Eur. 6.8, USP 32), incubation of the compounds were lasted for 120 min in SGDig. Following a deviation to a higher pH in medium, the most of global pharmacopoeias endorse a 45 min increase in the incubation to thoroughly evaluate the discharge of tested compounds. So consequently, both the non-capsular and capsular powder were encapsulated for 120 min in the FSIDig medium as bioactive compounds sustained to be discharged from the investigated capsule systems after incubation time of 45 min in it. At pre-determined time intervals (0 to 120 min SGDig and 0 to 150 min for those in FSIDig), the pH was sustained to 6.8 (initial value) for samples incubated in the FSIDig, whereas as for samples in the SGDig the pH did not fluctuate significantly, so no such pH adjustment was required. For analytical evaluations, an aliquot of 100 µL samples were taken with subsequent dilution with mixture (900 µL) of methanol/formic acid/bidistilled water (50/10/40, v/v/v) [38] to terminate any possible enzyme reaction and for stabilization of the polyphenols. Successively, the mixture containing polyphenols was undergone centrifugation (10,000 rpm for 5 min) (Centrifuge 5810/5810R, Eppendorf AG, Hamburg, Germany) to get the separated dispersed components. After centrifugation, supernatants from the mini Eppendorf centrifugation tubes were transferred to amber colored glass vials and kept at − 20 °C until analysis.

Acute toxicity

The acute oral toxicity study was carried out in compliance with Organization for Economic Cooperation and Development (OECD) guideline 425 [37]. The male albino mice (n = 21), divided into two groups i.e. control ($10 \times 1 = 10$) and treated ($11 \times 1 = 11$) intended for underway investigations were fasted 12 h prior consumption of dosage. During this period, weights of all mice were noticed and afterwards only treated group was given the dose in form of solution of microencapsulated products of microbial extracts (2000 mg/kg) by gavage. The control group was given the saline solution without the microbial extracts. After dosing, the mice were observed individually critically for first 30 min, intermittently for the first day (24 h), and recurrently subsequently for fortnight of feeding period for any possible toxicity symptoms, toxicity, gross behavioral changes, and/or mortality.

Preparation of cottage cheese with incorporation of microbial extracts and its nutritional and sensory evaluation

The cottage cheese was prepared and supplied on special request by the local cheese manufacturing company according to our prescribed instructions/recipes and without the addition of any additive and/or colorant and/or flavoring agent. Three groups of cottage cheese (each comprising 100 g) were designed in such way that (i) a group without the addition of any extract was taken as control (ii) cheese with the addition of best performing microencapsulated powder i.e. 5% WPHG + 5% pectin (T_A) (3 g/100 g cheese) and (iii) cottage cheese with microbial ethanolic free extracts (80 mg/mL).

The nutritional and sensory evaluation of the samples (control sample, cottage cheeses added with microencapsulated powder extracts and cottage cheeses added with microbial ethanolic free extracts) was performed right after the preparation (T_o) and after 7 days of storage (T_7) at 4 °C. The nutritional composition (ash, moisture, protein, fat, and carbohydrate) was calculated according to AOAC procedures [5]. The antioxidant capacity of samples was determined according to the DPPH assay described by Hameed et al. [22, 23]. Briefly, freshly prepared 0.3 mM solution of DPPH was mixed with 2.5 mL 5 mg/mL sample extracts and reaction mixture was kept in the dark for 20 min in glass tubes. The decreasing absorbance was measured at 517 nm to calculate the percentage inhibition of the radicals. Whereas the reducing power of the samples were measured according to above described CUPRAC assay. The sum of saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUSFAs) and polyunsaturated fatty acids (PUFAs) were determined by summing up their respective kind of fatty acids detected via by analyzing the petroleum ether extract previously obtained, by gas-chromatography coupled with a flame ionization detector (GC–FID), according to the procedure described by Reis et al. [44]. Sample color was measured in a colorimeter (model CR-400, Konica Minolta Sensing, Inc., Japan), using the illuminant C and a diaphragm aperture of 8 mm. The CIE L⁄a⁄b⁄ colour space values were recorded using the data software "Spectra Magic Nx" (version CM-S100W 2.03.0006). Three readings on the sample top and bottom parts, were used.

Cheese liking evaluation

In order to check whether the addition of free or microencapsulated extracts had any influence on the sensory scores of cheeses, a study was carried out comprising one hundred fourteen consumers (63 females and 51 males aged from 16 to 55 years old). They were conscripted rendering to their inclination to join in, and with the obligation of consuming cheese (at least one time a day) and not owing any kind of intolerance to cheese or cheese components. Three cottage cheese (each 100 g) were served to consumers: 'C' (control cottage cheese without additions of any extract), CapC (cottage cheese with added microcapsule powder), and 'FExC' (cottage cheese

added free extracts) without sharing any information about the contents of cheese. Consumers were instructed to write down their consuming experience of cottage cheese in relation to texture, global, and taste fondness by nine-point hedonic scales ranging from 1 = "dislike extremely" to 9 = "like extremely". For identification, the samples were coded with three-digit roman numbers codes and were served with a balanced design [33]. Between samples, consumers were given mineral water to rinse their mouth to further avoid any wrong sensory perception by consumers. All experiments were accomplished in submission with the local/national legislation, and according to the institutional framework and practices established University Ethics Committee. A written consent was received from each participant after the disposal of written information before the induction of each sensory evaluation activity.

Statistical analysis

All the experimental data has been repeated three times. All statistical analyses were conducted using a two-way analysis of variance with Dunnett's comparison tests or unpaired t-tests. These calculations were carried out using GraphPad Prism Software for Windows (GraphPad Software, San Diego, CA, USA). Significance was observed at $p < 0.05$.

Results and discussion

Physico-chemical properties of microencapsulated powders

The physico-chemical characterization is important crucial to understand the physico-chemical interaction that occurred between the extracts and the wall material which would help us to estimate the preservation of antioxidant capacity of microcapsules in food products [51]. The knowledge of hygroscopicity and solubility is essential to know about the powder stability and storage, reconstitution and, hence, bio-digestibility/bioaccessibility respectively. The physico-chemical properties of four kinds of microcapsule (T_A, T_B, T_C, and T_D) powders are shown in the Fig. 1. As can be seen in Fig. 1, the nature of wall material has significance influence on the physico-chemical properties and morphology of the resulting powders. Generally, microcapsules showed significantly higher ($p < 0.0001$) moisture contents and water activity (A_w) values than the control. Additionally, among the four treatments, all the microencapsulated powders exhibited considerable difference for moisture and A_w with the 5% WPHG + 5% pectin (T_A) and 5% PHGG and 5% pectin (T_C) showed highest moisture ($7.5 \pm 0.1\%$ and $6.2 \pm 0.1\%$ respectively) whilst at the same time T_A and 10% of PHGG (T_D) showed highest A_w (0.26 ± 0.01 and 0.27 ± 0.01 respectively) (Fig. 1a, b). The moisture and A_w range detected for all treatment was 5.5 to 7.5% and 0.14 to 0.27 respectively. All the encapsulated powders showed moisture contents and A_w below the threshold levels specified for microencapsulation by freeze drying [49]. So, the possible reason of higher moisture and A_w of microcapsule powders goes to adopted encapsulation methodology (i.e. freeze drying) for which many authors have also confirmed higher moisture contents and A_w compared to other methods of microencapsulation [16, 31]. The smaller pore size in the surface of wall material also hampers the mass transfer of moisture during sublimation at freezing temperature (i.e. −40 or −60 °C) which probably resulted in increase in water retention. The observed solubility of microcapsule powders varied from $70.44 \pm 1.4\%$ (T_D) to $93.4 \pm 1.01\%$ (T_A). The combination of 5% WPHG + 5% pectin (T_A) showed highest solubility values after control (Fig. 1c). The huge variation in the solubility trend of all microcapsule powders clearly bespeaks about the dependence of this parameter on the nature of wall material which means that microcapsule powders with high solubility index can be easily and effectively reconstituted in the water system for their application in food and pharmaceutical processing. Regarding hygroscopicity, a range of 4.9 ± 0.01 (T_B) to 12.4 ± 1.07 (T_C) was noticed (Fig. 1d), describing a low value for all encapsulated powders especially for T_B and TA. The low value of hygroscopicity is beneficial for the conservation and preservation of the bioactive components MC extracts. Despite the higher moisture contents, the lower hygroscopic values was also associated with the freeze drying method as larger particle size is found in microencapsulation by freeze drying compared to spray dried product [34]. Glass transition temperature (T_g) values are in a close relationship with stability of microcapsules while storage. Greater T_g values usually refers to greater stability of powders. The T_g of all powders were found in the range of 14.1 ± 0.3 (T_D) to 55.4 ± 1.2 (T_A) (Fig. 1e). The variation in the T_g of all treatments may be attributed to vast variations in the molecular weight and moisture contents of resulting powders as wall materials with higher molecular weights showed higher T_g previously [31]. Consequently, the treatments with combination of two wall materials (T_A and T_C) showed higher T_g than that of others (Fig. 1e).

Morphology and size distribution

In order to fully establish and apprehend the competencies of microcapsules with respect to their potential applications, it is also necessary to characterize the microcapsules with reference to their morphology and size distribution. The size of microcapsules is crucial in relation to their flowing and mechanical properties, shipment and adhesion to the surface. The microcapsules

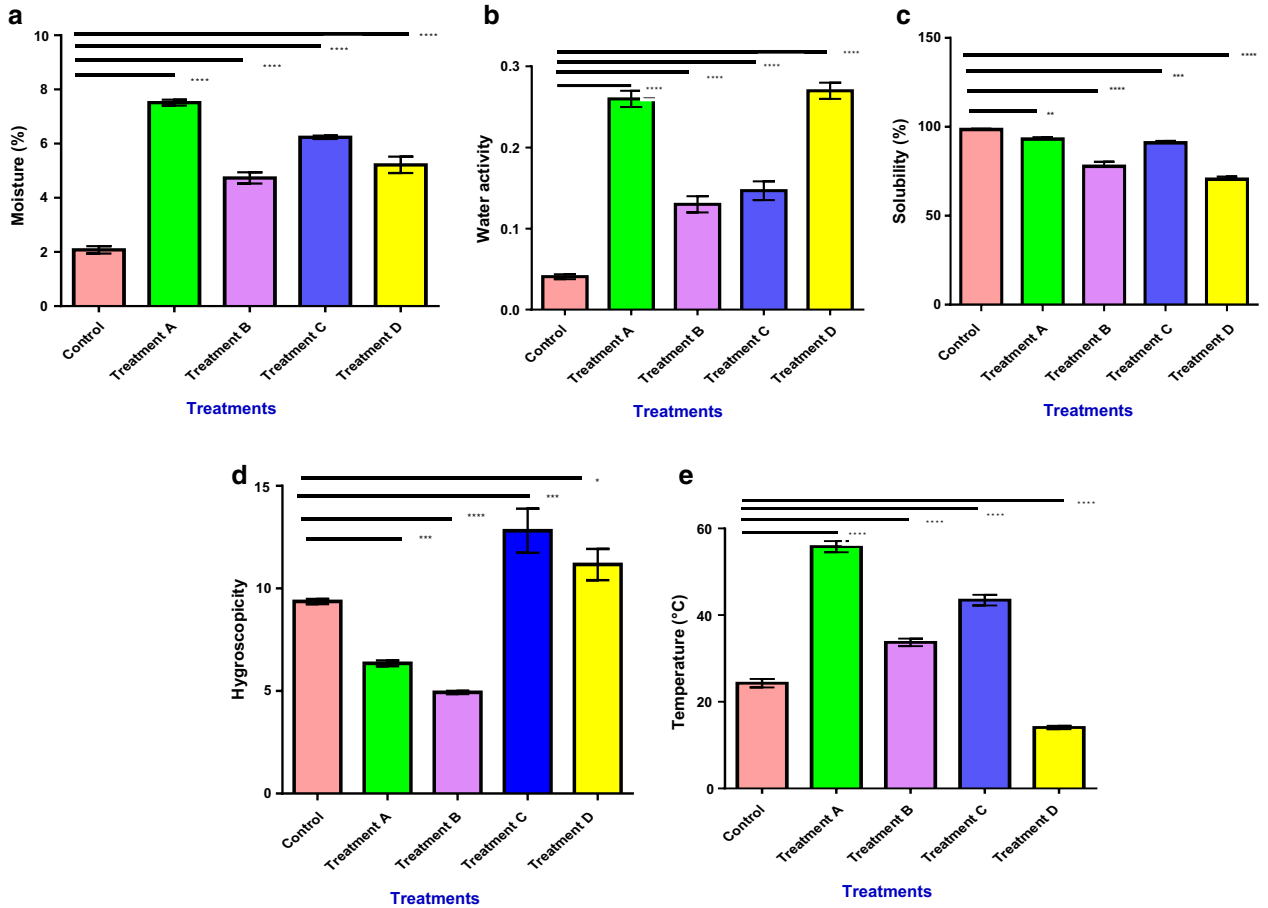

Fig. 1 Physico-chemical properties of microbial ethanolic extracts microencapsulated with Treatment A (T_A), Treatment B (T_B), Treatment C (T_C), and Treatment D (T_D) by freeze drying. Data is represented as mean of 3n ± S.D. **** Very extremely significant (p-value < 0.00001), *** extremely significant (0.00001 < p-value < 0.0001), ** very significant (0.0001 < p-value < 0.001), significant (0.001 < p-value < 0.01), and ns not significant

from each treatment showed varied surface morphology, size, and very dissimilar topography. Figure 2 showed the micrograph of microcapsule powders from each treatment. All the microcapsules exhibited non-ellipsoidal, slightly toothed morphology with rough surface devoiding any fissures, cracks or pores except T_D which showed deep depressions or/and pores in the surface. The non-existence of pores, as in first three treatments, is crucial for maintaining the stability and integrity of microcapsules, and an indicator for the high efficiency of microencapsulation process which ultimately warranty the safe and targeted delivery of bioactive compounds too. Whereas, the presence of pores usually permits the entry of oxygen and in turn facilitates the oxidation of bioactive compounds. Moreover, Table 1 represents the particle size distribution and average particle size of the four treatments. The diameter of freeze-dried microcapsules ranged from 12.06 to 42.75 µm. The observed diameter range was found less than the previous finding of other authors which may be due to manual excessive size reduction via mortar and pestle [11, 18, 51]. Additionally, the other possible reason of smaller diameter is the lack of agglomeration since the formation of irreversible bridges may lead to formation of larger particles [51]. This evidence of non-agglomeration is also guarantee longer shelf life of microcapsules even under accelerated storage conditions. With respect to span values, the observed span scores were ranged from 1.72 (T_D) to 2.28 (T_A). All treatments showed the span values lower than observed span values by previous authors for microencapsulation of plant extracts [18, 19]. These lower span values for microencapsulation of microbial antioxidants also symbolic to more homogenous particle distribution in the microcapsule powders of four treatments.

Flow properties of microencapsulated powders

All the microencapsulated powders showed regular powder flowing properties with non-significant ($p > 0.05$)

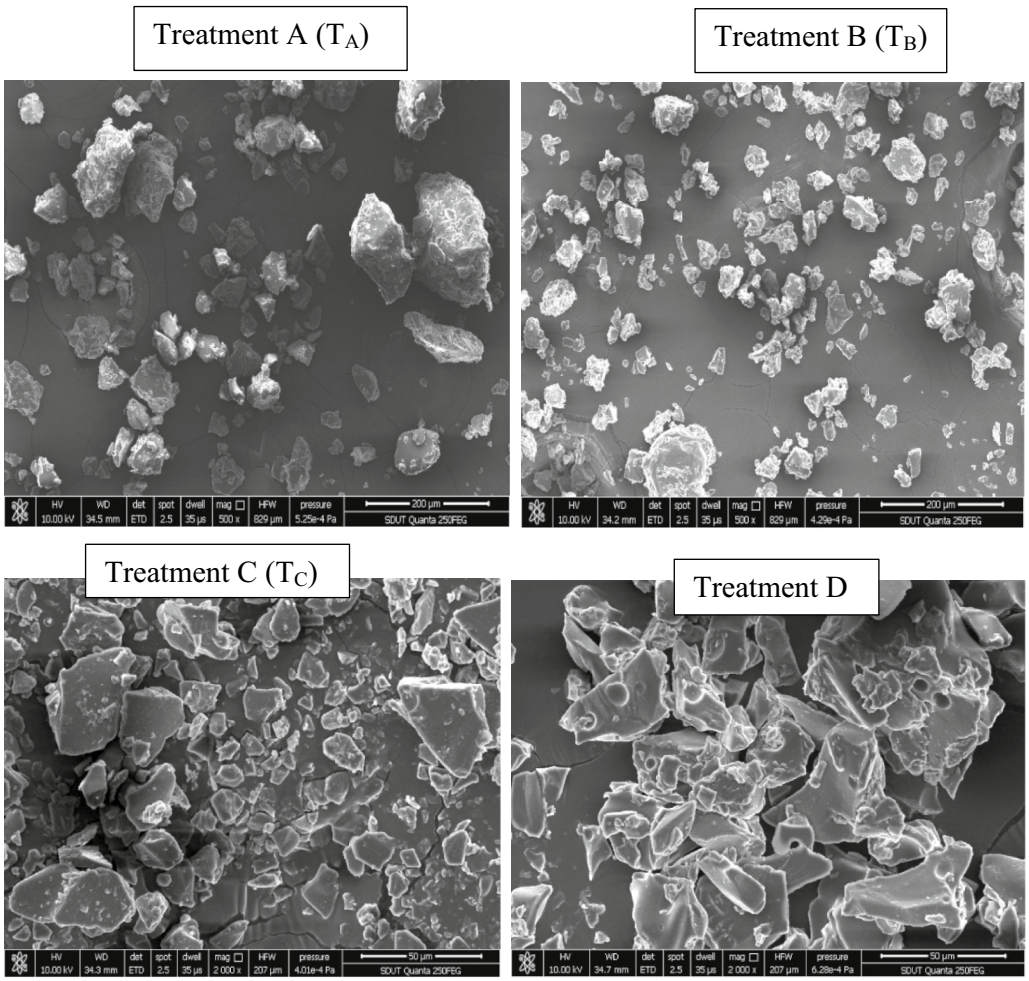

Fig. 2 SEM micrographs of microbial extracts microencapsulated with 5% WPHG + 5% pectin (Treatment A), 10% WPHG (Treatment B), 5% PHGG + 5% pectin (Treatment C), and 10% of PHGG (Treatment D) by freeze drying. Data is represented as mean of 3n ± S.D

Table 1 Average diameter and particle size distribution (Span) of the microbial extract microencapsulated with WPHG, pectin and PHGG and their combinations by freeze-drying method

Treatments	Average diameter (μm)	Span
T_A	12.06 ± 1.3	2.28 ± 0.2
T_B	42.75 ± 3.7	1.97 ± 0.07
T_C	21.80 ± 2.2	2.49 ± 0.1
T_D	25.36 ± 1.6	1.72 ± 0.3

Data is represented as mean of 3n ± S.D

variations among all the microencapsulated wall material treatments (Table 2). T_A and T_B showed highest bulk and particle density values which are highly desirable for safe product processing, transport and packaging activities the powders with high bulk density also offer less empty intra-particle available spaces for air or/and oxygen which means less probability of product degradation via oxidation [49]. The particle density of all microcapsules varied from 750 to 730 kg/m^3 which is an indication of identical porosity index in all microcapsule powders and existence of rough or/and toothed surfaced microcapsules [49]. The angle of repose of all powders was about 40–42° and similarly the percentage compressibility of all microcapsules was near 40% (Table 2). An angle of repose within the range of 40–45° was a good symbol of powders with desired flow characteristics and low Hausner ratio (0.9–1.1) were symbolic to powder viscosity having cohesiveness, an expressive of negligible poor flow with possible cohesive forces [6]. Regular surface morphology and large particles from freeze dried powders may be attributed towards the reduced Hausner ratio which resulted in improved flow properties with decreased cohesive forces [40]. The higher microencapsulation yield

Table 2 Flow properties of microbial extracts microencapsulated with 5% WPHG + 5% pectin (T_A), 10% WPHG (T_B), 5% PHGG + 5% pectin (T_C), 10% of PHGG (T_D) by freeze drying

Parameters	5% WPHG + 5% pectin (T_A)	10% WPHG (T_B)	5% PHGG + 5% pectin (T_C)	10% of PHGG (T_D)
Bulk density (kg/m^3)	137.5 ± 3.9a	135.2 ± 4a	132.8 ± 5.9a	132.2 ± 3.1a
Compact density (kg/m^3)	224.8 ± 3.1a	221.3 ± 3.4a	220.5 ± 5.3a	219.6 ± 4.9a
Particle density (kg/m^3)	747.5 ± 3.9a	740.7 ± 2.5a	739.3 ± 5.6a	737.6 ± 6.0a
Angle of repose (°)	42.6 ± 1.8a	41.6 ± 0.7a	43.3 ± 1.6a	42.2 ± 1.1a
Chroma	23.3 ± 1.1a	24.3 ± 0.7a	25.7 ± 0.5a	23.1 ± 0.9a
Compressibility (%)	42.2 ± 2.5a	40.5 ± 1.3a	41.2 ± 0.5a	39.5 ± 1.1a
Hausner ratio	0.93 ± 0.1a	1.1 ± 0.3a	0.97 ± 0.1a	1.13 ± 0.0a
Yield microencapsulation (%)	95.3 ± 1.7a	93 ± 1.2a	93.9 ± 2.2a	91.1 ± 1.1a

Data are expressed as 3n ± S.D. Same letters within the same row mean a non-significant difference ($p > 0.05$)

(> 90%) in all freeze-dried powders, especially with 5% WPHG + 5% pectin (T_A), showed excellent retention of microbial extracts during microencapsulation by freeze drying (Table 2). It is established fact that wall materials of WPHG or with combination of starchy/sugary material form a crust having reduced inner impact with limited convection heat transfer phenomenon, and consequently, minimum loss of extracts via volatilization or surface diffusion during subsequent operations. Some studies also suggested that there is no observed interaction between the protein hydrogels, used as wall material, and the polyphenol extracts so that kind of wall material could function as safe container with higher retain-ability and guaranteed protection [46].

Antioxidant components and antioxidative activities

The antioxidant components and bioactivities of freshly extracted pure ethanolic extract (control) and their lyophilized microencapsulated powders from four treatments (T_A, T_B, T_C, and T_D) are shown in Fig. 3. With respect to TPC, all the microencapsulation treatments showed significantly less ($p < 0.0001$) TPC compared to control except T_A whose TPC were found to be non-substantially different from the control (Fig. 2a). Among the treatments, only T_A was found to retain significantly higher contents ($p < 0.05$) of phenolic compared to T_B, T_C, and T_D whereas non-significance retain-ability of TPC was detected while making comparison among T_B, T_C, and T_D. Almost same trend was detected, while measuring the TFC and TCT of control and four treatments as observed in TPC calculations, except in TCT where TCT of T_A was weakly but significantly lower than that of control (Fig. 3c). The bioactivities of control (pure ethanolic extract) and four treatments showed a bit different tendency than the bioactive components. The bioactivities of all microencapsulated powders were significantly ($p < 0.0001$) lower than that of control. The main reason of low bioactivities of all microencapsulated powders might be due to loss and degradation of polyphenolic compounds during microencapsulation and re-extraction of polyphenolic extracts from powders. In β-carotene bleaching and CUPRAC assays, not only all the walled material powders showed less bioactivity than that of control but substantial variations also existed among all the four treatments, however, in chelating assay, a non-significant variation was noticed between control and TA. According to results, 5% WPHG + 5% pectin (T_A) showed the maximum retain-ability (> 80%) of the microbial antioxidant components and their bioactivities followed by T_C (5% PHGG and 5% pectin). So, microencapsulation of microbial bioactive extracts with 5% WPHG + 5% pectin can be a viable option for protecting microbial bioactive components of MC against degradation during the processing and storage.

In-vitro digestion, targeted and control release, and bio-accessibility studies of microencapsulated powder

Polyphenols mostly exist in their stable cationic form after gastric digestion, and converted and degraded to their aldehyde, acidic, or ketonic (i.e. ketonic hemiketal, quinonoidal, and chalcone) forms in intestinal phase to higher pH [13]. This transformation and annihilation of polyphenols at intestinal level is one of the main reasons of their low bioavailability at intestinal phase. Therefore, it was of highly desirable and likely to check either microencapsulation of pH sensitive microbial antioxidants with the best-found whey protein isolate hydrogel + pectin combination (T_A), was a good strategy in delivering the pH thermo-labile microbial bioactive compounds in their most active form (cationic) at intestinal phase. For this purpose, microbial polyphenols released from microcapsules (T_A) were measured from simulated intestinal fluid, in order to establish the fact that whey protein hydrogel + pectin (T_A) can be considered an appropriate

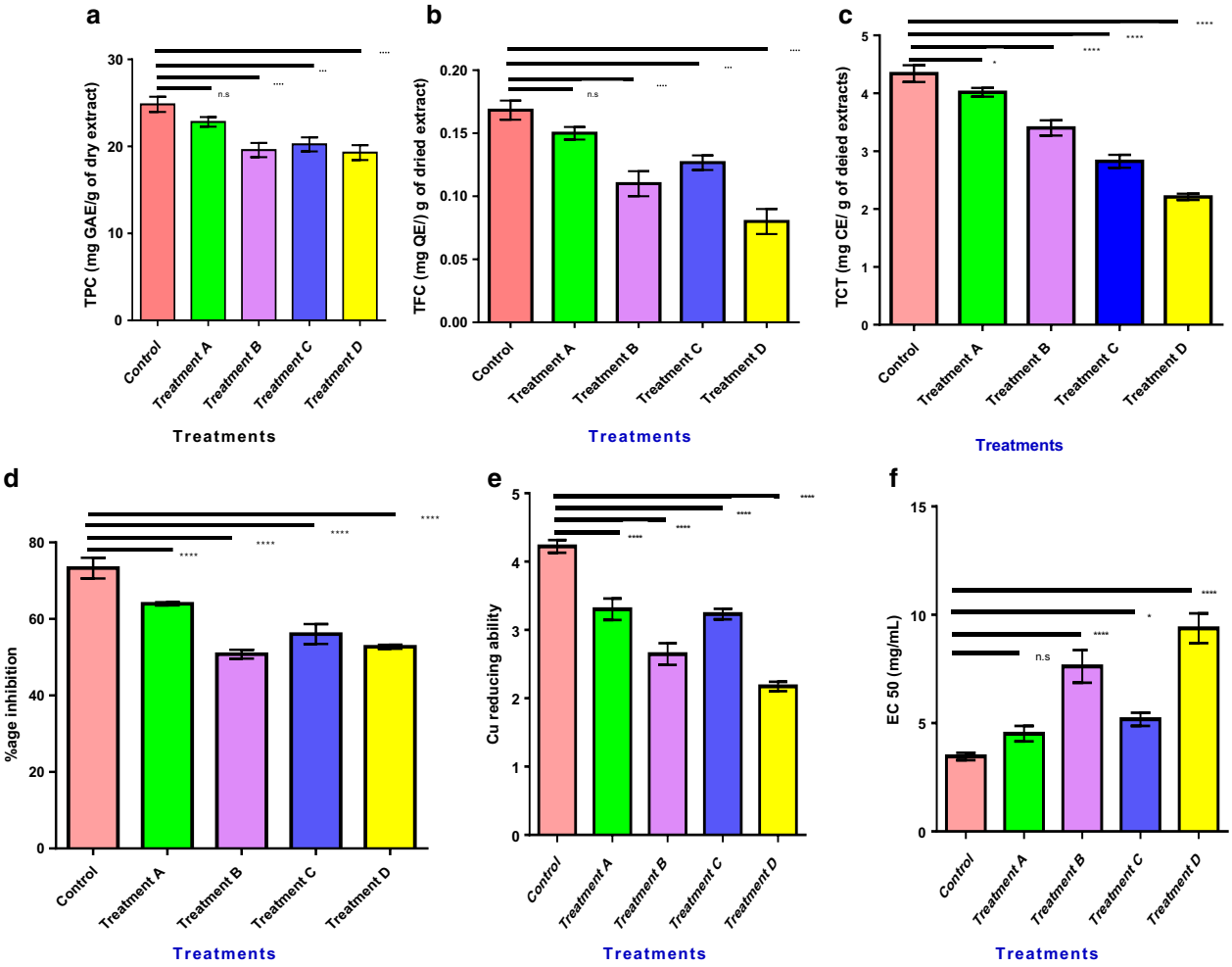

Fig. 3 Antioxidant components and antioxidant activity of free ethanolic extracts (control) and microcapsules powders encapsulated with Treatment A (T_A), Treatment B (T_B), Treatment C (T_C), and Treatment D (T_D) by freeze drying. Results expressed as 3n ± S.D. TPC based on calibration curve of gallic acid, expressed as mg gallic acid equivalents (GAE) per gram of dry extract; r = 0.993. TFC expressed as mg quercetin equivalents (QE) per gram of dry extract; r = 0.9891. TCT content based on calibration curve of (+)-catechin, expressed as mg catechin equivalents (CE) per gram of dry extract; r = 0.991. The values β-carotene bleaching assay of selected extracts (mg/mL) based on percent bleaching inhibition. CUPRAC of selected fungal extracts (mg/mL) based on absorbance (λ450 nm). EC_{50} values (mg/mL) is representative of the effective concentration at which 50% of Fe^{2+}/Cu^{2+} ions were chelated. **** Very extremely significant (p-value < 0.00001), *** extremely significant (0.00001 < p-value < 0.0001), ** very significant (0.0001 < p-value < 0.001), significant (0.001 < p-value < 0.01), and ns not significant

matrix for stabilizing polyphenols at severe intestinal conditions. Pure and freshly extracted ethanolic extracts and non-gelled mixed whey protein–microbial extract solution (matrix solution) were incubated as reference samples (Fig. 4). The pure and freshly extracted ethanolic extracts were also impregnated with the microcapsule powders to calculate the relative polyphenolic concentration. This relative polyphenolic concentration was maximum (~6000%) at t = 0 and taken as refence for the subsequent measurements of stability and release kinetics of microencapsulated and non-microencapsulated extracts in digestion media. At gastric phase, most of polyphenols found to exist in their stable cationic form [13], thus after incubation of freshly extracted mycelial ethanolic extracts of MC at pH 1–2, negligible annihilation of polyphenols happened and polyphenolic concentration stayed constant at 75–85% of total phenolic contents (Fig. 4). Moreover, the relative polyphenolic concentration of microencapsulated powders and gelled matrix released polyphenolic extracts doubled within the initial 30 min of incubation. This considerable increased in the relative polyphenolic concentration might be dedicated to time-dependent protection of wall material and subsequent release of polyphenolic rich extracts with

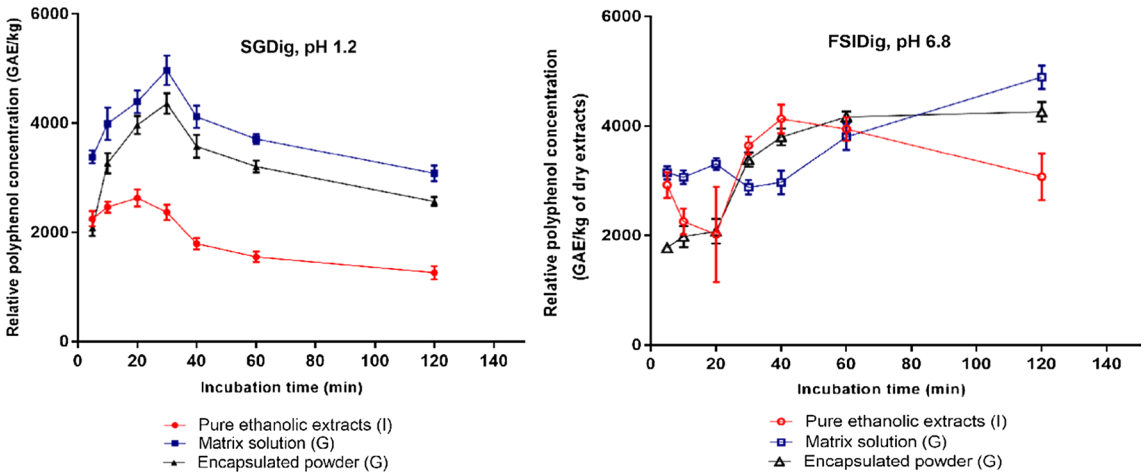

Fig. 4 Time-dependent relative polyphenol concentration in simulated gastric fluid without enzyme (SGDig, pH 1.2) (closed symbols) and fed-state simulated intestinal fluid with pancreatin (FSIDig, pH 6.8) at 37 ± 1 °C (open symbols) after incubation of the free ethanolic microbial extract (circles), the mixed non-gelled whey protein–microbial ethanolic extract solution (matrix solution) (cubes) and the corresponding microbial ethanolic extract-loaded microcapsules (triangles). The maximum TPC was noted from the initial 0.7% polyphenols concentration (set as reference) in release media of all samples which was around 6000 GAE)/kg of dry extract. This TPC value was set as maximum measured TPC at incubation t = 0 and all the latterly measured polyphenolic concentration (from incubation t = 0 to t = 120 min) were described as relative polyphenolic concentration to this maximum limit. The incubation in SGDig and FSIDig was carried out independently, not consecutively. Data are given as 3n ± confidence interval at 95%

minimal exposure to digestion media. However, it was not the same case with non-encapsulated pure extracts due to which its relative polyphenolic concentration dropped enormously.

In intestinal phase, the severe pH condition (6.8) lead to degradation of almost 30% of ethanolic polyphenolic extracts during the very first 5 min of incubation especially in case of pure ethanolic extracts. So, a trend of continuous and sudden degradation of remaining polyphenols from intentionally added ethanolic extracts was noticed. After 120 min of incubation, only 30% of initially added polyphenolic contents of ethanolic extracts were left. There was seen an increase in the relative polyphenolic concentration from 20 to 90 min both in microencapsulated and intentionally added pure ethanolic extracts. However, this increased in relative polyphenolic concentration was more in pure ethanolic extracts but could not sustain after 90 min of incubation while microencapsulated extracts showed a sustainable and continually increasing relative polyphenolic concentration after 20 min which surpassed the relative polyphenolic concentration of pure ethanolic extracts after 90 min (Fig. 4). The increase in the relative polyphenolic concentration of pure ethanolic might be due to degradation of polyphenolic compounds into secondary polyphenolic compounds which might act as pro-antioxidants. The sustainable and continually increasing relative polyphenolic concentration of microcapsules might envisage the important role of microencapsulation of extracts in timely and targeted delivery of core extracts. Of note, the relative polyphenolic concentration of matrix solution (reference) was of highest from 40 min to onward as compared to relative polyphenolic concentration of pure ethanolic extracts and microcapsules which might be due to better provision of shield by non-gelled matrix to extracts. In addition, the degradation rate of polyphenols was less than the release rate of polyphenols from microcapsules during the first 40 min of incubation in intestinal pH. As a result, a substantially higher amount of polyphenolic compounds could be achieved in FSIDig over the investigated time period after the incubation of microcapsule containing extracts compared to pure ethanolic extracts. A higher concentration of polyphenols at intestinal phase is synonymous to higher resorption and hence its bioavailability. However, right after the release from microcapsules, only 20% released polyphenolic contents found to face the fate of degradation due to which only 80% of initially added polyphenolic were measured from intestinal fluids. Comparatively, at gastric phase (pH 1.2), degradation of polyphenolic compounds occurred during the first 20 min in pure ethanolic extracts, and in case of microcapsules, the degradation rate of polyphenols was higher than the release rate of polyphenolic compounds during the first 20 min (Fig. 4). At gastric phase, all polyphenolic compounds released from microcapsules after 20 min with less degradation rate afterwards.

With respect to matrix solution (reference), no degradation was noticed while incubation of non-gelled matrix solution at gastric level (pH 1.2) throughout the investigated time period. Conversely, almost 30% of initially available polyphenols lost during the incubation in FSI-Dig (pH 6.8), but this degradation of polyphenols began after the 20 min (with no initial observed degradation) of incubation which continued afterwards up to 40 min (Fig. 4). The addition of extracts/microcapsules into the intestinal fluid did not change the pH of medium (pH 6.8) which is indication of stabilization of polyphenolic compounds entrapped in the combinations of whey protein isolate and pectin at pH 6.8. These findings are in line with previous findings of various authors who described the interaction of polyphenolic compounds with different native proteins from neutral to alkaline pH a warranty to substantially slower down the degradation rate of entrapped bioactive compounds than in the protein free solution [1, 25, 42, 54]. However, in whey proteins + pectin based microcapsules, the whey protein is thermally crosslinked and thus no or at least less interaction between native proteins and polyphenols occur. As many studies already demonstrated that addition of polysaccharide (i.e. pectin) capable of preventing/mitigating the protein-polyphenolic interaction [32, 50]. Therefore, the observed stabilizing effect in the FSIDig, can be referred to diffusive and time based release of stable polyphenols from the microcapsules.

Acute toxicity

The microbial lipids production by MC have been commercialize since last three decades and still hold the "GRAS (Generally recognized as safe)" status [41] since no metabolite and lipids from MC showed any toxicological sign for human consumptions as trial were carried out by United States Food & Drug regulatory authority and other European regulatory authorities [41]. The commercial production of orange-yellow food grade natural colorant (β-carotene) from MC is in its developmental stage also considered safe for human consumption [14]. Since the utilization of very same mycelia of MC, grown under the same fermentation conditions as for lipids and β-carotene production, for natural antioxidant production so one can established the fact that natural antioxidant production from MC would be safe too for application in food and pharmaceutical industry. To further strengthen this hypothesis acute oral toxicity studies were carried out with albino (male) mice (n = 21). In vivo toxicity appraisal is the initial toxicological evaluation of medicinal plant/marine/microbe derived products by histopathology and oral acute toxicity studies. In vivo toxicity outcomes will be important to conclusively judging the safety of medicinal plant/microbial derived products. However, acute toxicity studies do not detect the effects of plant/marine/microbe derived products on central nervous system, cardiovascular, and pulmonary/respiratory systems, and must be evaluated separately prior declaring safe for human consumption. Moreover, there are still some concerns related to extrapolating the animal data to human, but studies showed mice give more credible prediction for human acute lethal dose than rats [52]. All the experiments were preceded according to strict guidelines of OECD [37]. The albino mice ($11 \times 1 = 11$) were orally administered with best declared microcapsule (T_A)/crude ethanolic extracts and were monitored (for any toxic sign and mortality) from very start after the oral administration till the end of specified period as mentioned above. Compared to controls ($10 \times 1 = 10$),

Table 3 The observations of general appearance, behavior, organ-to-body weight index, and biochemical parameters of serum for control and treated groups of albino mice

Observations	Control group[b]	Treated group[c]
General observations[a]		
Skin and fur	Normal	Normal
Eyes	Normal	Normal
Mucous membranes	Normal	Normal
Behavioral changes	Normal	Normal
Salivation	Normal	Normal
Urination (color)	Normal	Normal
Lethargy	Normal	Normal
Sleep	Normal	Normal
Diarrhea	Not found	Not found
Coma	Not found	Not found
Tremors	Not found	Not found
Organ-to-body weight index[1] (%)		
Kidney	1.11 ± 0.17	1.53 ± 0.23[a]
Heart	0.57 ± 0.07	1.23 ± 0.2[a]
Liver	4.8 ± 0.39	6.87 ± 0.47[a]
Lung	0.79 ± 0.04	1.53 ± 0.11[a]
Spleen	0.67 ± 0.09	1.77 ± 0.1[a]
Body weight	22.7 ± 0.8	24.7 ± 1.3[a]
Biochemical parameters of serum		
TBIL (μmol/L)	1.2 ± 0.2	0.9 ± 0.1
ALT (U/L)	17.7 ± 4.4	18.1 ± 5.1
AST (U/L)	81.4 ± 3.7	79.1 ± 5.2
ALP (U/L)	86.3 ± 9.6	87.7 ± 11.3

[a] General observations were taken through the treatment period started right after oral administration of specific doses

[b] Control groups (treatment without crude extract)

[c] Treated groups (treatment with 2000 mg/kg crude extract)

[1] Organ body index = (organ weight × 100)/body weight; microcapsule (T_A)/crude ethanolic extracts was administered to mice at a dose of 2000 mg/kg; values are mean ± SD (n = 21) at 5% level of significance (*p < 0.05)

Table 4 Antioxidant activity, nutritional and color parameters evaluation of (a) control cottage cheese in comparison with cottage cheeses enriched with (b) free microbial extracts, and (c) microencapsulated extracts

Parameters	Control cottage cheese		Cottage cheese with free extracts		Cottage cheese with microencapsulated extracts	
	0 day	7th day	0 day	7th day	0 day	7th day
Antioxidant activity*						
DPPH radical scavenging activity	> 180	> 180	83.1 ± 8.7b	124.2 ± 4.4a	111.8 ± 3.5a	73.7 ± 6.4c
Reducing power	145 ± 4.9	129.3 ± 7.5	76.7 ± 5.3c	113 ± 6.1a	91.6 ± 5.3b	63.5 ± 4.8d
Nutritional values						
Ash (g/100 g fw)	1.5 ± 0.07	1.68 ± 0.06	1.33 ± 0.03	1.86 ± 0.08	1.58 ± .08	1.45 ± 0.05
Fat (g/100 g fw)	16.2 ± 0.06	15.9 ± 0.1	15.58 ± 0.04	16.78 ± 0.1	16.54 ± 0.13	17.03 ± 0.1
Protein (g/100 g fw)	12.3 ± 0.04b	12.1 ± 0.08b	12.6 ± 0.04a	12.2 ± 0.1a	12.3 ± 0.05ab	12.6 ± 0.12ab
Carbohydrates (g/100 g fw)	1.15 ± 0.09	1.97 ± 0.2	1.25 ± 0.04	2.04 ± 0.2	1.24 ± 0.11	1.72 ± 0.15
Energy (kcal/100 g fw)	204.9 ± 1.3a	204.3 ± 0.8a	194.8 ± 2.4a	217.1 ± 1.7a	191.3 ± 1.7b	201.1 ± 2.1b
SFA (relative %)	68.3 ± 0.3b	71.1 ± 0.4b	73.4 ± 0.3a	71.7 ± 0.6a	70.6a ± b	71.6 ± 1.2b
MUFA (relative %)	26.3 ± 0.4a	24.2 ± 0.3a	22.3 ± 0.2b	23.4 ± 0.1b	25.5 ± 0.11a	24.7 ± 0.17a
PUFA (relative %)	5.3 ± 0.13	4.5 ± 0.4	4.6 ± 0.34	4.6 ± 0.05	4.7 ± 0.07	4.8 ± 0.2
Color parameter						
*L**	91.3 ± 0.43	91.4 ± 0.39	92.5 ± 0.25	90.1 ± 0.75	91.5 ± 0.26	90.4 ± 0.3
*a**	2.4 ± 0.11a	2.1 ± 0.12a	1.7 ± 0.0b	1.5 ± 0.07b	1.8 ± 0.02b	1.6 ± 0.04b
*b**	9.6 ± 0.08b	11.3 ± 0.2b	10.23 ± 0.14ab	12.31 ± 0.41ab	11.6 ± 0.34a	11.5 ± 0.11a

Antioxidants activity was expressed in EC_{50} (mg/mL). *L**, *a**, and *b** represents the color parameters. In each row, different letters (a–d) correspond to significance ($p < 0.05$) among the treatments whereas data without letter is considered non-significant. Results are expressed as 3n ± S.D

no obvious signs of distress, toxicity, and mortality were observed at a dose of 2000 mg/kg by gavage. Physically, body, eyes, fur, and skin were normal without significant variations in behavior, food intake and water consumption. The body weight of treated mice exhibited non-significant rise as compared to control, which evidenced the negligible toxicity on growth of mice (Table 3). Generally, the alternation of body weight and internal organ weight is indicator of possible toxicity, and unwanted variations in the physiological, metabolic, and pathophysiological index respectively. Generally, a decrease (≥ 10%) in body weight (from initial weight) is symbolic to adverse effect of a drug/product [43]. Furthermore, statistically, weak significant increase in absolute and relative organ-to-body-weight was witnessed (Table 3) which needs to be investigated separately. Following organ weight index, hematopoietic system serves as an important index of the pathological and physiological status [2]. After 14 days of oral administration of microcapsule (T_A)/crude ethanolic extracts, no substantial variation in the serum levels of total bilirubin level (TBIL), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were detected, hence, confirming the non-toxic nature of microcapsule (T_A)/crude ethanolic extracts (Table 3).

Cottage cheese with and without microencapsulated microbial extracts

Table 4 comprehensively describes the outcomes of cottage cheese prepared with free and micro-capsular extracts in comparison with control cheese. The in vitro bioactivity of cheese in terms of antiradical potential and reducing power revealed that incorporation of free microbial extracts resulted in higher antioxidant power initially (t = day 0) which progressively decreased up to 7th day due to possible degradation of antioxidants. In case of microcapsules, the bioactivities of cheese was at lower at the beginning (t = day 0) which reached its peak value on the last day (t = day 7) of analysis. This later rise in antioxidant capacity can be credited to protection aided from microencapsulation together with sustained release pattern.

With respect to nutritional and color evaluation, the incorporation of either free or microencapsulated microbial extracts did not produce substantial variations in the ash, carbohydrate, fat, and polyunsaturated fatty acids (PUFA) contents of cottage cheese (Table 4). However, a weakly significant amount of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) was observed in both treated (free extract and microcapsule added) groups of cottage cheese. These slightly higher contents of SFA and MFA might be dedicated to the shield offered

Table 5 Sensorial appraisal of cottage cheese by selected panel of consumers

Sensory attributes	Control cottage cheese	Cottage cheese with free extracts	Cottage cheese with microencapsulate extracts
Texture liking	8.06 ± 0.9	7.8 ± 0.5	7.2 ± 0.4
Flavour/taste liking	7.9 ± 0.8	7.2 ± 0.4	7.06 ± 0.2
Overall liking	7.16 ± 0.4	6.8 ± 0.2	6.93 ± 0.1

In each row, different letters (a–d) correspond to significance ($p < 0.05$) among the treatments whereas data without letter is considered non-significant. Results are expressed as 3n ± S.D

by the added extracts in cottage cheese against the degradation. However, all the nutritional values obtained were found to be in the range of reference values for this kind of cottage cheese. In general, the incorporation of extracts (either free or microencapsulated) introduced slight (but non-significant) variations in the profile of protein, SFA and MUFA only when compared with control cottage cheese. In the appraisal of color, the addition of extracts failed to produces marked variations in L^* and a^* while comparing with control group.

Sensory appraisal

The impact of adding free or microencapsulated microbial extracts in cottage cheese on consumer liking was assessed. The best declared microcapsule (whey protein + pectin) was chosen for this analysis. The consumers' appraisals were summarized in Table 5. The simple ANOVA results clearly depicted no obvious variations in the liking values of three kinds of cottage cheeses. The consumers literally did not perceive any kind of difference regarding taste/flavor affecting the liking of cottage cheeses. Consumers rated the three kinds of cheeses alike with reference to taste/flavor liking. Hence the addition of free or microencapsulated microbial extracts did not brought about the variations in the consumers expected and actual physical and sensorial cues.

This work concluded the possibility of microencapsulating the microbial ethanolic extracts of MCWJ11 with the combinations of selected biopolymers. 5% WPHG + 5% pectin (T_A) showed higher scores with respect to physico-chemical, powder flowing, retention of bioactive compounds, and hence encapsulating properties over the other three treatments. The in vitro digestibility assays of microcapsule powder (T_A) showed improved, targeted and time-dependent release of bioactive compounds with minimal degradation and hence loss even under adverse digestion conditions. Acute oral toxicity experimentation with albino mice confirmed the non-toxic nature of microcapsule (T_A)/crude ethanolic extracts at a dose of 2000 mg/Kg since no aberrant variation in general appearance, behavior and biochemical parameters of serum were observed. The fortification of cottage cheese with microencapsulated microbial extract produced non-significant variations with respect to cottage cheese with added free microbial extracts in physico-chemical, nutritional, compositional, and sensory analysis terms.

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Authors' contributions

YS supervised this work. AH and SAH performed all the experimental work. AH wrote this manuscript. HARS edited and reviewed the whole manuscript and provided suggestions to main authors about overall research plan and antioxidant activities. ZM, SN, SU and YW assisted in the formulation of cottage cheese and their subsequent analysis. All authors read and approved the final manuscript.

Conflict of interests

Authors declare no competing interests.

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