Glutathione – enriched baker's yeast: production, bioaccessibility and intestinal transport assays

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Running title: investigation on GSH-enriched yeast

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ABSTRACT

Aims: a glutathione (GSH) yeast-based biomass (*S. cerevisiae*) was used to investigate GSH stability, solubilization during gastrointestinal digestion and GSH intestinal transport.

Methods and results: a post-growing procedure was applied to improve intracellular GSH yeast content. The presence of adenine (ADE) in the biotransformation solution (CYS-GLY-GLU mixture) and alternatively, a glucose shot after 4 h incubation, allowed to obtain cells containing about GSH 1.6-1.7 % dcw (dry cell weight) (control 0.5%). Yeast samples were subjected to *in vitro* gastro-intestinal digestion and absorption assays employing Caco-2 and HT29-MTX cell lines in different proportions (100/0, 70/30 and 50/50). Trials were also performed to verify intestinal cell viability.

Conclusions: at least 87% of ingested GSH is available in reduced form for intestinal absorption. *In vitro* GSH transport assays indicated that GSH is poorly absorbed (<20%). Nevertheless, studies in response to oxidative stress induced by H_2O_2 demonstrated a protective role of the GSH-enriched biomass towards intestinal cell viability.

Significance and Impact of Study: an enriched GSH yeast-based biomass has been obtained using a post-growing procedure. Although GSH present in enriched yeasts is poorly absorbed by intestinal cells, this biomass showed an intestinal local protective effect, improving cells viability when a simulated oxidative stress was applied.

Keywords: Glutathione, yeast, Caco-2, HT29-MTX, gastrointestinal digestion, intestinal absorption.

INTRODUCTION

Glutathione (GSH) is a tripeptide consisting of L-glutamate, L-cysteine and glycine. It is found in millimolar concentrations (0.2-10 mM) in all cells, from prokaryotes to eukaryotes, and is the most abundant low molecular thiol in biological systems (Anderson 1998; Ault and Lawrence 2003; Wen et al. 2005; Wang et al. 2007). Intracellularly, GSH is kept in its thiol form by GSSG reductase, a NADPH-dependent enzyme (Anderson 1998). GSH is committed to many physiological processes, however its functions may be summarized in three main topics: antioxidant, immunity booster and defense molecule (Li et al. 2004). These characteristics make GSH an important biochemical drug for the treatment of numerous diseases, such as HIV infections, liver cirrhosis, gastrointestinal and pancreatic inflammation, as well as neurodegenerative diseases (Townsend et al. 2003; Li et al. 2004). Cellular GSH concentration is markedly reduced in response to protein malnutrition, oxidative stress, and many pathological conditions such as Crohn's disease, atherosclerosis and diabetes (Li et al. 2004; Wu et al. 2004). Moreover, studies evidenced that GSH may be therapeutically effective when given in high doses to depleted subjects (Perricone et al. 2009).

Currently, *Saccharomyces cerevisiae* and *Candida utilis* are the most commonly used microorganisms for GSH fermentative production on industrial scale, with a GSH content of 0.1-1% dcw (dry cell weight) (Li et al. 2004). They are rich sources of protein, soluble fiber, minerals (Ca, P, K, Mg, Cu, Fe, Zn, Mn and Cr) and B vitamins, often recommended as a dietary supplement (Yamada and Sgarbieri 2005; Bekatorou et al. 2006). In previous reports (Rollini et al. 2011; Musatti et al. 2013) we focused on the set up of a post-growing procedure (biotransformation) for GSH accumulation employing commercial baker's yeast (*S. cerevisiae*), while most of the published papers concentrated on GSH accumulation in growing cells (Li et al.

2004; Wen et al. 2005; Wei et al. 2008; Liang et al. 2009; Nisamedtinov et al. 2010). The use of a post-growing procedure employing commercial baker's yeast, a low cost cell source available on the market, represents an alternative strategy to traditional GSH accumulation inside yeast cell, easy and feasible for an industrial up-scale, in particular for nutraceutical applications.

In order to design an adequate dietary supplementation, it is necessary to evaluate if a supplement remains intact after the digestion processes and if it is absorbed by the epithelium to reach the systemic circulation. The *in vitro* combination of a simulated gastrointestinal digestion and a cellular model of intestinal epithelium to emulate absorption has been demonstrated useful to evaluate the aspects related to the processes of solubilization and absorption of a compound in the gastrointestinal tract, and hence to study the bioavailability (Au and Reddy 2000). The term "bioavailability" refers to the fraction of an ingested compound that is solubilized during gastrointestinal digestion and reaches the systemic circulation (Calatayud et al. 2010). Cellular models derived from colorectal cancers (Caco-2 and HT29-MTX) have provided a useful tool for these studies. These cell types have conserved parts of the program of epithelial differentiation, expressing many genes of differentiated intestinal epithelial cells and maintaining in turn, the ability to form monolayers of polarized cells once they have reached confluence in culture (Hidalgo et al. 1989; Lesuffleur et al. 1990).

GSH bioavailability has been studied *in vivo* by several authors but up to now with discordant results. Hagen et al. (1990) and Rahman and MacNee (1992) affirmed that plasma GSH concentration in rats increased from approximately 15 to 30 μM after oral GSH administration, indicating that oral supplementation may be useful to enhance GSH tissue availability. Likewise, Aw et al. (1991) reported that in mice oral GSH intake can increase GSH concentrations in several tissues following its depletion. On the contrary, Witschi et al. (1992) reported that in

humans, systemic GSH availability is negligible and it is not possible to increase circulating GSH to a clinical beneficial extent by an oral administration of a single dose of 3 g.

The present study was aimed at obtaining a GSH - enriched *S. cerevisiae* biomass and investigating GSH solubilization and stability during gastrointestinal digestion. GSH absorption by intestinal epithelium *in vitro* models was also analysed, and trials were also performed to verify whether GSH supplementation may be a useful strategy to improve intestinal cell viability.

MATERIALS AND METHODS

Biotransformation conditions

Samples of commercial baker's yeast (*S. cerevisiae*) in compressed form, identified as Fala (Lesaffre, Trecasali-Parma, Italy) were employed. Fresh yeast cells (i.e. 1 day of storage time) were suspended (5% dcw) in 10 mL of a biotransformation solution (identified as CYS-GLY-GLU mixture) containing the three GSH precursor amino acids and the following compounds (g l^{-1}): glucose 80, sodium citrate 10, ammonium sulphate 7, monobasic potassium phosphate 3.5, magnesium sulphate 0.5, cysteine 4, glycine 4, glutamic acid 4. The reaction mixture was incubated at 28 °C and 200 rpm. CYS-GLY-GLU mixture alone or added with adenine (ADE, 0.5 g l^{-1}), adenosine (ADO, 1.5 g l^{-1}), dithiotreitol (DTT, 3 g l^{-1}) or a combination of ADO/DTT or ADE/DTT were tested, as well as a further glucose (50 g l^{-1}), after 4 h incubation with CYS-GLY-GLU mixture with or without ADE (Table 1). Aliquots were collected at the beginning and after 24 h incubation and then analysed for intracellular GSH contents.

Lyophilization

Yeast samples were lyophilized in order to faithfully reproduce the form usually marketed. Cells were suspended in distilled water (20% dcw), placed in stainless steel trays as a thin layer, and then frozen at -40 °C for 4 h. Cell dehydration was carried out at 25 °C and 1.33 Pa for 30 h in an Edwards Minifast MFD 01 lyophilizer (UK), until a maximum residual humidity of 5–8% was reached.

Simulation of a gastrointestinal digestion. Bioaccessibility studies

GSH standard solutions at two different concentrations (40 and 200 mg l^{-1}, equivalent to 0.13-0.65 mM) were employed for bioaccessibility tests, as well as one GSH-enriched yeast sample obtained from the biotransformation experiments described above (trial 4, GSH 1.63 ± 0.15% dcw), and one commercial baker's yeast in compressed form (control, 0.45 ± 0.05% dcw). Yeast samples were both lyophilized before use.

Cell culture grade water (B. Braun Medical S.A., Spain) was used throughout the *in vitro* digestion assay. Enzymes and bile salts for *in vitro* gastrointestinal digestion were all purchased from Sigma: porcine pepsin (enzymatic activity 944 U mg^{-1} protein), porcine pancreatin (activity equivalent to 4×US Pharmacopoeia specifications/mg pancreatin) and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts). The study was carried out using the simulated digestion process proposed by Laparra et al. (2003) modified for a yeast biomass.

Samples (1-3 g for yeast biomass, and 40 and 200 mg l^{-1} for GSH solutions) were weighed, 25 ml of cell culture grade water was added and pH adjusted to 2.0 with 6 M HCl. Freshly prepared pepsin solution (10% w v^{-1} pepsin in 0.1 M HCl) was added to provide 2 mg pepsin g^{-1} sample. Sample was made up to 30 ml with cell culture grade water, and incubated in a shaking water bath (120 strokes min^{-1}) at 37 °C for 2 h. Gastric digests were then raised up to pH 6.0 by

drop-wise addition of 1 M $NaHCO_3$. The pancreatin-bile extract mixture (0.4% w v^{-1} pancreatin and 2.5% w v^{-1} bile extract in 0.1 M $NaHCO_3$) was added to provide 0.5 mg pancreatin g^{-1} sample and 3 mg bile extract g^{-1} sample, and the incubation at 37 °C continued for additional 2 h. After the intestinal digestion step, pH was adjusted to 7.2 by drop-wise addition of 0.5 M NaOH. Digests were then transferred to polypropylene centrifuge tubes and centrifuged at 10000 rpm for 30 min at 4 °C to separate the soluble (bioaccessible) fraction. All trials were performed in triplicate. GSH and GSSG contents were evaluated in the obtained bioaccessible fractions.

Intestinal cell maintenance

GSH transport through the intestinal epithelium was investigated using Caco-2 cell line and co-cultures of Caco-2 and HT29-MTX cells. Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC, number 86010202, Salisbury, UK). Cell maintenance was performed in 75 cm^2 flasks to which 10 ml of pH 7.4 Dulbecco's modified Eagle's medium (DMEM) with 4.5 g glucose l^{-1}. DMEM was supplemented with 10% (v v^{-1}) fetal bovine serum, 1% (v v^{-1}) non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (N-2-hidroxyethylpiperazine-N'-2-ethanosulfonic acid), 100 U penicillin ml^{-1}, 0.1 mg streptomycin ml^{-1}, and 0.0025 mg amphotericin B ml^{-1} (DMEMc).

HT29-MTX cells were kindly provided by Dr. T. Lesuffleur (Institut National de la Santé et de la Recherche Médicale, INSERM UMR S938, France) (Lesuffleur et al. 1990). Cells maintenance was performed in 25 cm^2 flasks to which 5 ml medium were added, consisting of DMEM at pH 7.4 containing 4.5 g glucose l^{-1} and supplemented with 10% (v v^{-1}) fetal bovine serum, 100 U penicillin ml^{-1}, 0.1 mg streptomycin ml^{-1}, 0.0025 mg amphotericin B ml^{-1} and 1 mM sodium pyruvate (HT-DMEMc).

Both cell lines were incubated at 37 °C, 5% CO_2 and 95% relative humidity atmosphere, changing the medium every 2–3 d. When cell monolayer reached 80% confluence, cells were detached by mean of a trypsin solution (0.5 mg l^{-1}) containing 0.22 g EDTA l^{-1}, and subsequently reseeded at a density of 5×10^4 cells cm^{-2}. All reagents used were purchased from PAA Laboratories GmbH (Germany).

Throughout the study, Caco-2 cell cultures were used between passages 20 and 35, while HT29-MTX cells were used between passage 40 and 50.

GSH transport assays: determination of apparent permeability coefficients (P_{app}) and percentage of transport

Transport tests were performed in 6-well plates equipped with a polyester membrane porous support (diameter 24 mm, pore size 0.4 μm; Transwell®, Costar Corp, USA); this insert separates the well into an apical (upper) and a basolateral (lower) compartment. Cells were seeded (5×10^4 cells cm^{-2}) on the apical side to produce monolayers of Caco-2, and of Caco-2/HT29-MTX (70/30 and 50/50 proportions). Subsequently, 1.5 ml medium (DMEMc for Caco-2 and HT-DMEMc for co-cultures) were added to the apical chamber and 2 ml to the basolateral one. Cells were incubated at 37 ºC, 5% CO_2 and 95% relative humidity, changing the medium every 2–3 d until cell differentiation was attained (13-15 d post seeding). During cell growth and differentiation in Transwell®, cell monolayer integrity was assessed daily from the sixth post-seeding day onward by measuring the transepithelial electrical resistance (TEER) using a Millicell®-ERS (Millipore Corporation, Madrid, Spain).

Transport assays were first performed employing GSH standard solutions (3 and 10 mM), as follows: 1.5 ml of the standard solution prepared in Hank's balanced salt solution (HBSS, PAA)

supplemented with 20 mM pH 5.5 o-2-(N-morpholine) ethanesulphonic acid (MES, Sigma, Spain) were added to the apical compartment, and 2 ml of HBSS with 10 mM pH 7.2 HEPES to the basolateral compartment. For the evaluation of the apparent permeability coefficients (P_{app}), at established timepoints (15, 30, 60, 120, 180 and 240 min), 300 µl were removed from the basolateral compartment and replaced with an equal volume of fresh medium (HBSS with 10 mM HEPES). GSH determination was carried out in the aliquots obtained at each time point as well as in the apical residual medium collected at the end of the experiment.

As regards the transport studies using bioaccessible fractions resulting from the gastrointestinal digestion, GSH standards (3 and 10 mM) or GSH-enriched yeast samples were employed. These samples were first inactivated by heating for 4 min at 100 ºC to inhibit protease activities and then cooled by immersion in an ice bath. Glucose (final concentration 5 mM, Sigma) was then added to facilitate cell viability. If necessary, NaCl (10 mM, Panreac) was used to adjust the osmolarity to 310 ± 10 mOsm kg^{-1} using a freezing point osmometer (Automatic Micro-Osmometer Type 15 Löser, Löser Messtechnik, Germany). Aliquots of 1.5 ml of the inactivated bioaccessible fraction supplemented with 20 mM pH 5.5MES was added to the apical chamber and 2 ml of HBSS supplemented with 10 mM pH 7.2 HEPES to the basolateral compartment.

The apparent permeability coefficients (P_{app}) were calculated from Equation 1:

$$P_{app} = (dC\ dt^{-1}) \times (V_r\ A^{-1}\ Co^{-1}) \qquad \text{(Eq. 1)}$$

where:

($dC\ dt^{-1}$) is the flow ($\mu M\ s^{-1}$) determined by the linear slope of the equation that governs the variation in the concentrations of GSH, corrected by dilution, against time;

V_r is the volume of the basolateral compartment (2 ml);

A is the surface occupied by the cell monolayer (4.67 cm^2);

C_o is the initial GSH concentration in the apical compartment (μM).

Besides the calculation of the apparent permeability, the rate of transport of bioaccessible fractions of GSH standards (3 and 10 mM) and GSH-enriched yeasts was also evaluated, performing transport assays for 2 h, without taking aliquots at different timepoints. For quantification of GSH transport, GSH contents were determined in the acceptor (apical) and donor (basolateral) medium collected at the end of the experiment. The percentages of transport to the basolateral compartment were calculated with respect to the initial quantity of GSH added.

During GSH cell transport trials, cell monolayer integrity was evaluated by measuring: a) TEER at several incubation times, including the start and end of the experiment, and b) P_{app} of the paracellular transport marker lucifer yellow (LY), added at 100 μM concentration to the apical compartment in the control and in GSH treated cells. Fluorescence of LY transported to the basolateral compartment was measured with a fluorescence microplate reader (PolarSTAR OPTIMA, BMG-Labtech, Germany) at excitation/emission wavelengths of 485/520 nm. To evaluate any possible interactions of LY with GSH uptake and transport, parallel experiments were performed with and without paracellular marker, which demonstrated the absence of interferences. The limits established for considering that the cell monolayer integrity was maintained were: TEER between 80% and 120% with respect to the control; LY $P_{app} \leq 0.5 \times 10^{-6}$ cm s^{-1}.

Effect of GSH on viability of intestinal epithelial cells

Cell viability assays were performed employing sodium resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma). The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and dihydroresorufin, measurable by colorimetric methods. This conversion is intracellular, facilitated by mitochondrial, microsomal and cytosolic oxidoreductases (O'Brien et al. 2000; Rocha et al. 2011).

Caco-2 cells were seeded at a density of 6.25×10^4 cells cm^{-2} in 96-well plates for 5 d and subsequently exposed for 1 h at 37 °C to GSH standard solutions (3 and 10 mM, prepared in HBSS) or GSH-enriched yeast (after *in vitro* digestion and thermal inactivation). Afterwards, 2, 10 and 20 mM H_2O_2 (Panreac) were added to cells and incubated for further 2 h. Additionally, cells without pre-treatment with GSH (solution or enriched-yeasts) were exposed to 2, 10 or 20 mM H_2O_2.

After exposure, the medium was withdrawn and 150 µl resazurin solution (10 µg ml^{-1} in MEM) were added. Well plates were incubated for 2 h at the same conditions. 100 µl for each reaction mixture were transferred to a 96-well plate and resazurin reduction was measured colorimetrically (570 and 600 nm) using a microplate reader (PolarSTAR OPTIMA).

Analytical procedures

Determination of intracellular yeast GSH was carried out as reported by Musatti et al. (2013). Briefly, samples were centrifuged (10600 g, 10 min) and collected cells were washed with distilled water, suspended in 0.5 g ascorbic acid l^{-1} and then thermally treated at 100 °C for 10 min in order to open cell structure and release GSH. After cooling in ice bath, samples were

centrifuged (10600 g, 10 min) to eliminate cell residues and intracellular GSH of supernatant fractions were determined.

GSH and GSSG identification and quantification were carried out by HPLC (Merck-Hitachi L-7000 System) equipped with a UV detector (210 nm), using a Purospher® RP-18 endcapped column (250 mm x 4 mm, Merck). The elution was performed with 25 mM NaH_2PO_4 (pH 2.8, flow rate 0.3 ml min^{-1}) at 30 °C. GSH and GSSG concentrations were calculated with respect to a calibration curve of aqueous standards of GSH and GSSG (5-50 mg l^{-1}). Standard GSH and GSSG were purchased from Sigma and HPLC-grade water was obtained through a Milli-Q A10 Gradient System (Millipore Corporation, USA).

Statistical Analysis

One factor analysis of variance (Microsoft Office Excel 2003) was applied to detect: i) differences in the GSH intracellular content (% dcw) among biotransformation solutions, ii) viability differences of Caco-2 cells. Duncan test was applied to detect means differences among the tested conditions. Differences were considered significant for $p<0.05$.

RESULTS

Biotransformation trials

Trials were performed by suspending yeast (5% dcw) in a biotransformation solution containing glucose and the three precursor amino acids involved in GSH synthesis (CYS-GLY-GLU), as previously reported (Rollini et al. 2011). Nevertheless, since GSH biosynthesis is ATP-dependent, some ATP-related molecules were also added for comparison purposes (Liang et

al. 2010; Musatti et al. 2013). Direct ATP addition was not chosen due to its high cost; instead, adenine (ADE) and adenosine (ADO), that can be considered as ATP precursors, were tested. Trials were also performed by adding dithiotreitol (DTT), an ATPase inhibitor.

As reported in Figure 1, all tested conditions led to a significant ($p < 0.05$) GSH increase respect to control samples with only the CYS-GLY-GLU mix (trial 1, GSH level 0.98% dcw). Glucose added was always consumed by the yeast in the first 3-4 h incubation, leaving at the end of the reaction a residual concentration of 5 mg glucose/L (HPLC detection limit). High GSH level was evidenced in the presence of 0.5 g ADE l^{-1} (trial 4, GSH 1.63% dcw), with about a 170% increase. The incorporation of ADO, led a limited GSH increase (about 115%, from 0.98 to 1.11% dcw, trial 2). The presence of DTT only inhibited GSH production when ADE was incorporated (trial 6). The addition to the reaction mixture of further 50 g glucose l^{-1} after 4 h incubation (trial 7, GSH 1.67% dcw) increased intracellular GSH, similarly to what observed when ADE was present in the medium (trial 4). However, glucose addition when ADE was present (trial 8) did not cause further GSH increase.

In vitro *gastrointestinal digestion*

Trials were first performed employing GSH standard solutions (40 and 200 mg l^{-1} comparatively). Results related to GSH and GSSG content at the beginning of incubation (t_o) and after the gastric and intestinal steps are reported in Figure 2. At the highest concentration (200 mg l^{-1}) a very limited amount of GSH (up to 10%) was oxidized to GSSG, while an oxidation of approximately 25% took place when 40 mg GSH l^{-1} were used. This oxidation mainly occurred after the intestinal digestion phase and may be due to the temperature (37 °C) maintained for 4 h and/or the neutral pH of intestinal stage.

Trials were also performed employing untreated and GSH-enriched yeasts, both in lyophilized form, with a GSH content of 0.5 and 1.6% dcw, respectively. At the beginning of the digestion (t_0) about 90% of the total GSH was found bioaccessible and this value increased to 98% at the end of the digestion (data not shown). Previous trials of gastrointestinal digestion on baker's yeast in compressed form (not lyophilized) showed that GSH bioaccessibility was only about 20% (data not shown). Thus, the lyophilization process applied contributed to increase GSH bioaccessibility. It is known that lyophilization process damages yeast cell structure in a way that GSH can be easily released (Musatti et al. 2013).

Gastrointestinal digestion tests produced a very limited GSH oxidation (Figure 3), up to 13% in the control yeast sample and to 7% in the case of GSH-enriched yeast. These results confirm what previously evidenced for GSH standard solutions.

GSH transport assays: determination of P_{app} and percentages of transport

In all transport assays performed the TEER and LY permeability values were maintained within the limits established for considering the integrity of the monolayer to be intact.

The P_{app} indicates the rate at which a compound passes across the intestinal cell monolayer and is a useful parameter for comparative studies, since values are adjusted by the surface of the monolayer, exposure time and concentration of the used compound. The P_{app} values obtained after 240 min exposure to GSH standard solutions were as follows: 9.9×10^{-7} cm s^{-1} for 3 mM and 1.2×10^{-6} cm s^{-1} for 10 mM. Using co-cultures of Caco-2/HT29-MTX (50/50 and 70/30), the P_{app} values for a 10 mM GSH solution were found similar to those obtained in Caco-2 monocultures alone (8.1×10^{-7} and 7.0×10^{-7} cm s^{-1} for 50/50 and 70/30, respectively).

Table 2 shows the percentage of GSH transported through Caco-2 or co-culture monolayers after 120 min exposure to either the bioaccessible fractions of GSH standard solutions (3 and 10

mM) and of enriched-yeast samples (GSH ≈ 3 mM). Transport rates were generally low (2.2-7.9%) and remained unchanged in presence of yeast. The incorporation of HT29-MTX cells to the monolayer produced a significant GSH transport increase, with values reaching 7% of the GSH initially added, in both standards and yeasts.

Effect of GSH on intestinal epithelial cells viability

Trials were performed to elucidate the possible protective role of GSH and GSH-enriched yeast on Caco-2 cell viability exposed to an oxidant (H_2O_2). As expected, Caco-2 cells viability decreases after H_2O_2 exposure, and this decrease was found H_2O_2 concentration-dependent. Figure 4 reports the results obtained by adding GSH-enriched yeast (1 h contact time) before H_2O_2 addition. For comparison purposes, pre-treatments with 3 and 10 mM standard GSH solutions were also performed. To be noted that a control test with yeast not containing GSH could not be performed because all *S. cerevisiae* strains have a physiological GSH content.

Results showed that pre-treatment with GSH-enriched yeast reduced intestinal cell death caused by H_2O_2. Future studies will verify if lower GSH levels in yeast would furnish the same results here reported and will be aimed at investigating if other yeast components (i.e. folates) may be involved, in association with GSH, in reducing oxidative stresses.

DISCUSSION

The yeast *S. cerevisiae* is one of the most studied microorganisms, widely used in traditional fermentation processes like wine, beer and bread making, but nowadays it also has numerous applications in other industrial productions such as bioethanol, production of enzymes and is

employed as dried-yeast for food supplement (Yamada et al. 2005; Bekatorou et al. 2006). It has been shown that the use of yeast favours the bioavailability of certain supplements; for example, selenium bioavailability in a selenium-enriched yeast increases up to 135%-165% (approximately 1.5-fold) in terms of tissue selenium content and 105%-197% (up to 2-fold) in terms of GPx activity, compared to selenite (Yoshida et al. 1999). The aim of the present study was to obtain *S. cerevisiae* with a high GSH content for a new food supplement formulation and to investigate the fate of the ingested GSH.

Applying a post-growing procedure to a commercial baker's yeast, already available on the market, has increased its GSH intracellular content. This procedure represents an alternative strategy easy and feasible for an industrial up-scale, to the traditional growth-related GSH accumulation. Commercial baker's yeast generally has a GSH content of about 0.5% dcw; owing to a biotransformation procedure with CYS-GLY-GLU mixture a GSH content of about 1% dcw is reached. The addition of adenine to the mixture or glucose, after 4 h incubation, increases GSH content up to 1.7% dcw, a three-fold increase respect to the physiological yeast GSH content. The proposed product falls within the description proposed by the Directive 2002/46/EC and the subsequent Regulation 1170/2009. This biomass can be considered a concentrated source of protein, soluble fibre, minerals and B vitamins as well as a concentrated GSH source. The maximum GSH daily supplementation in Italy, 50 mg, can only be achieved after ingestion of 3 g of the proposed yeast.

Yeast samples were lyophilized to faithfully reproduce the dose form usually marketed. The lyophilization process, as demonstrated in this study, facilitates GSH solubilization during its passage through the gastrointestinal tract, and therefore increases its availability for further absorption through the intestinal epithelium. In addition, gastrointestinal digestion does not negatively affect GSH levels, only inducing a limited oxidation (7-13%). Therefore it can be

concluded that 87-93% of ingested GSH should be available for its absorption in the reduced form.

There are various *in vitro* models for evaluating intestinal absorption in humans, although the one that is most widely used is the Caco-2 cell line. This cell line, derived from colon adenocarcinoma, differentiates spontaneously in culture, producing a monolayer of epithelial cells which shares many of the morphological and functional characteristics of mature enterocytes (Hidalgo et al. 1999). There are studies showing that Caco-2 cells express numerous transporters of the human small intestine, and therefore they are considered a good model for the evaluation of intestinal transport mechanisms (Maubon et al. 2007). This model is currently the most commonly employed for *in vitro* study of absorption of pharmaceuticals and nutrients. Numerous studies demonstrate that it is possible to correlate the apparent permeability coefficients obtained in Caco-2 cells with the magnitude of *in vivo* absorption, although the suitability of this correlation depends on the nature of the transport of the compound. In the present work we took into account the study conducted by Yee (1997), according to which a compound with a P_{app} in Caco-2 monolayers $< 1\times10^{-6}$ cm s^{-1} has low *in vivo* absorption (0–20%), one with a P_{app} between 1 and 10×10^{-6} cm s^{-1} has moderate *in vivo* absorption (20–70%) and one with a $P_{app}>10\times10^{-6}$ cm s^{-1} has high absorption (70–100%).

The P_{app} values obtained in the present study classify GSH as an element with a low *in vivo* absorption (<20%) at pH 5.5, typical value of the duodenum. This parameter was not found to be modified when Caco-2/HT29-MTX co-cultures were used, model which more realistically emulates the intestinal wall where both cell types (absorptive cells and mucus secreting cells) coexist in variable proportions depending on the part of the intestinal tract. Furthermore, results indicate that GSH present within an enriched yeast biomass is transported similarly a GSH standard solution.

Although GSH supplementation does not substantially increase its systemic levels, GSH presence was found beneficial towards intestinal cells viability. Especially in a form of an enriched biomass, GSH could protect intestinal cells when these are subjected to oxidative stress, here simulated by the use of H_2O_2. The results of the present study demonstrate an increase in viability of cells treated with enriched yeasts when a situation of oxidative stress is induced. Gastrointestinal tract is one of the major target for oxidative stress damage due to the constant exposure to diet-derived oxidants, mutagens, and carcinogens as well as to endogenously generated reactive oxygen species (Couto et al. 2012). Thus, ingested GSH may have local protective effect, acting together with GSH coming from the biliary efflux.

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Table 1. Scheme of the biotransformation trials performed with Fala yeast: (1), control: samples containing the CYS-GLY-GLU mixture; (2) to (8), samples added with (g l^{-1}) adenosine (ADO), dithiotreitol (DTT), adenine (ADE), combinations ADO/DTT and ADE/DTT, as well as glucose shot at 4 h incubation.

Trials	**ADO**	**DTT**	**ADE**	**Glucose**
(1) control	-	-	-	-
(2) + ADO	1.5	-	-	-
(3) + DTT	-	3.0	-	-
(4) + ADE	-	-	0.5	-
(5) + ADO + DTT	1.5	3.0	-	-
(6) + ADE + DTT	-	3.0	0.5	-
(7) control + glu	-	-	-	50
(8) + ADE + glu	-	-	0.5	50

Table 2. GSH transported (respect to the initially added, %) from apical to the basolateral compartment in Caco-2 cells and co-cultures Caco-2/HT29-MTX after 120 min exposure to the bioaccessible fractions of standard GSH solutions (3 and 10 mM) and of GSH-enriched yeast (mean ± standard deviation, n=3).

Cell line	**Transported GSH (%)**		
	Std 3 mM	**Std 10 mM**	**Yeast**
Caco-2	2.16 ± 0.06	2.33 ± 0.18	2.23 ± 0.05
Co-culture 70/30	3.25 ± 0.31	3.35 ± 0.33	3.92 ± 0.36
Co-culture 50/50	6.96 ± 0.40	7.58 ± 0.25	7.93 ± 0.84

FIGURE LEGENDS

Figure 1. Intracellular GSH increase (% respect to control sample – trial 1) obtained after 24 h incubation in different biotransformation conditions (mean ± standard deviation, n=3). Letters (a-c) on multiplot bars indicate significant differences (Duncan test, $p < 0.05$) in GSH increase among the tested biotransformation conditions (1:control; 2:+ADO; 3:+DTT; 4:+ADE; 5:+ADO+DTT; 6:+ADE+DTT; 7:control+glu; 8:+ADE+glu).

Figure 2. GSH (■) and GSSG (■) concentration (mg l^{-1}) at different steps of the *in vitro* gastrointestinal digestion (t_0 and after gastric phase and complete gastrointestinal digestion) related to GSH standard solution of 40 (A) and 200 (B) mg l^{-1} (mean ± standard deviation, n=3). On multiplot bars, lower (a-b) and upper-case (A-B) superscript letters indicate significant differences (Duncan test, $p < 0.05$) in GSH and GSSG concentration, respectively, among gastrointestinal digestion phases in trials performed with 40 mg l^{-1} GSH standard solution (Figure 2A). Lower (c-e) and upper-case (C-E) superscript letters indicate significant differences (Duncan test, $p < 0.05$) in GSH and GSSG concentration, respectively, in trials performed with 200 mg l^{-1} GSH standard solution (Figure 2B).

Figure 3. GSH (▨) and GSSG (▩) concentration (mg l^{-1}) (means, n=3) at different steps of the *in vitro* gastrointestinal digestion (t_0 and after gastric phase and complete gastrointestinal

digestion) related to lyophilized control yeast (GSH 0.45 ± 0.05% dcw) (A) and GSH-enriched yeast (GSH 1.63 ± 0.15%) (B).

Figure 4. Viability (% respect to control sample ■) of Caco-2 cells exposed to different pretreatments (1 h with 3 ■ or 10 ■ mM GSH solution or with GSH-enriched yeast ■), and subsequently treated with H_2O_2 (2-10-20 mM) for 2 h (mean ± standard deviation, n=5). Control sample (■) refers to untreated cells prior H_2O_2 exposure. Different superscript letters (a-d) indicate significant differences (Duncan test, $p < 0.05$) in cell viability among pretreated (or not, in the case of control) cells exposed to different H_2O_2 concentration.