The **aim of this project** was to develop functional foods based on specific edible mushroom extracts able to effectively reduce cholesterol levels in serum by acting simultaneously at two different levels:

(I) reducing endogenous cholesterol synthesis by inhibiting the hydroxymethylglutaryl-CoA reductase (HMGCR, the key enzyme in the cholesterol metabolism) and

(II) impairing exogenous cholesterol absorption during digestion by its displacement from the dietary mixed micelles, by scavenging of bile acids during the intestinal digestion to avoid their reabsorption stimulating the hepatic transformation of cholesterol bile acids and by inhibiting the pancreatic lipase (PL).

Results: A large number of wild and cultivated mushrooms were screened to identify those with the ability of acting as (I) and (II). Three mushroom species were selected, *Agaricus biporus* (white button mushroom), *Pleurotus ostreatus* (Oyster mushroom) and *Lentinula edodes* (Shiitake mushrooms) and they were submitted to standard and advance extraction technologies such as accelerate solvent extraction (ASE) and supercritical fluid extraction (SFE) to obtain fractions enriched in HMGCR inhibitors (F1) and PL inhibitors (F2), fractions enriched in water-insoluble high-molecular-weight polysaccharides (β-glucans and chitins) (F3) and fractions enriched in fungal sterols (ergosterol, fungisterol etc.) (F4).

Water extracts or extracts obtained by ASE using water as pressurized solvent at 10.7 MPa and 25ºC as common extraction conditions and 5 cycles of 5 min each for *P. ostreatus* fruiting bodies and 15 cycles of 5 min for *L. edodes* showed very interesting HMGC inhibitory activities (60 – 80% inhibition). However, the highest inhibitory activities found in *A. bisporus* mushrooms were observed in their methanol:water (1:1) extracts (obtained by standardized or ASE extractions). Accelerated solvent extractions (10.7 MPa, 25ºC, 5 cycles of 5 min) were more effective in the extraction of F1 fractions than supercritical CO_2 extractions using 9 MPa, 40ºC with 10% ethanol as modifier. These results suggested that the potential HMGCR inhibitors were different in the selected mushroom species.

Previous publications pointed lovastatin as the compound responsible for the HMGCR inhibitory activity detected in these mushrooms. However, after HPLC-DAD and HPLC-MS/MS analysis of the samples with inhibitory activity, no statins were found. Results suggested that the responsible compounds were proteoglucans of molecular weight higher than 10 kDa in *A. bisporus*, higher than 3 kDa in *P. ostreatus* and lower than 3KDa (but not eritadenine) in *L. edodes*. They were only partially resistant to an *in vitro* digestion model but their bioavailability in Caco2 cell cultures could not be confirmed because of the test sensibility limits.

In vitro enzymatic kits to detect PL inhibitors pointed a few mushroom species (*Lepiota procera, grifosa frondosa, Pleurotus eryngii, Lyophillum shimeji, Morchella conica, Marasmius oreades*) with PL inhibitory capacity (equivalent to a 25% of an orlistat concentration). However, when their PL inhibitory activity was measured using an *in vitro* digestion model no inhibition was detected. Moreover, fractions (F2) obtained with ASE (using water and ethanol as pressurized solvents) from the most promising mushroom specie (*P. eryngii*) showed no significant PL inhibitory activity.

Polysaccharide (PSC) enriched fractions (F3) were obtained by ASE and compared with a standardized PSC extraction method. Pressurized water extracted more polysaccharides than ethanol. Extraction yields were also higher in individual more than sequential extractions being the optimal extraction parameters: 200ºC, 5 cycles of 5 min each at 10.7 MPa. The high molecular weight (>3.5 kDa) water-insoluble fraction, isolated from the ASE fractions contained mainly β-

glucans and in lower concentrations chitins and α-glucans. The ASE extracted fractions showed different PSCs profile depending on the mushroom strain considered. When compared with the standard PSC extraction method, ASE extraction methods were better suitable for *L. edodes* and *A. bisporus* than for *P. ostreatus*.

The obtained PSC fractions (with both standard and ASE extraction methods) were able to scavenge bile acids during an *in vitro* digestion model when applied in a ratio similar to a cereal PSC extract bearing the claim of being able to reduce cholesterol levels and containing mainly β-glucans (ratio bile extract: PSC (1:100 w/w)). As expected, the fungal PSC extracts were not absorbed by Caco2 cells as they are classified as indigestible fibers.

Ergosterol and its derivatives could be extracted by ASE from *A. bisporus* fruiting bodies but also from their by-products (the lower parts of the stipes discarded during harvesting). The highest amount of sterols (per gram of mushroom dw) were extracted with pressurized ethanol (10.7 MPa), ratio 1:4 (sample: sand) and 5 cycles of 1 min at 100ºC or 5 cycles of 5 min at 50ºC. However, SFE could also be utilized to obtain sterol enriched fractions (F4) using CO_2 and 10% ethanol as co-solvent (30%) or only CO_2 (55%) at 40ºC and 30 to 9 MPa.

Ergosterol and the SFE extracts were able to displace the cholesterol from the dietary mixed micelles (DMM) during an *in vitro* digestion model of cholesterol-enriched lards when mixed at ratios similar to β-sitosterol (1:2 (cholesterol: ergosterol)). When the DMM fractions were isolated and applied to Caco2 cell cultures to study intracellular transport, 10 fold higher levels of cholesterol were found in the basolateral compartment than of fungal sterols.

A few functional foods were prepared by mixing food products (liquid yogurt, spreadable cheese, liver pâté, croquettes and muffins) with a specific water extract from *L. edodes* with HMGCR inhibitory capacity, with a specific β-glucan-enriched extract from *P. ostreatus* and with an ergosterol-containing extract from *A. bisporus* in order to study their stability through the technological processing and bioavailability as bioactive ingredients inside a real foodstuff. The HMGCR inhibitory activity was completely lost if the processing included heating (pasteurization at 90ºC, frying at 160ºC or baking at 200ºC), β-glucans and ergosterol were more resistant to heat treatments but their levels were lower in the liver pâté, thus addition to already processed yogurt or cheese was encouraged. Spreadable cheese functionalized with F1, F3 and F4 partially resisted an *in vitro* digestion model but their bioavailability could not be measured using Caco2 cells because if applied at subtoxic concentrations they were below the threshold of the detection.

Conclusion, potential socio-economic impacts and use: In principle, double targeting functional foods able to reduce cholesterol levels in serum can be designed by supplementations (after heat treatments) of food products with a mixture of specific mushroom extracts enriched in HMGCR inhibitors proteoglucans, β-glucans and fungisterols. Thus, the new functional foods have similar hypocholesterolemic effects than those already known for phytosterols + cereal β-glucans + statins but all combined in one product. However, *in vivo* studies in animals are needed in order to ensure their bioavailability. Thus, at the present and thanks to the knowledge acquired with this project, a new research project is being developed to carry out those animal tests, to study expression of genes involved in the cholesterol metabolism and to further characterize the nature of the proteoglucans with HMGCR inhibitory activity. Moreover, another project has been recently granted to scale up the β-glucans extraction process from mushrooms to industrial levels for exploitation of results by three enterprises.