Tools to develop dairy ingredients: bioactive and preservative purposes

Dora Khouja

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Supervisors

Doctor Sandrina Heleno

Doctor Lillian Barros

Doctor Khalil Zaghdoudi

Bragança

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Abstract

High cholesterol levels are a major concern these days due to the serious illnesses associated with this problem. Interesting progress has been made to overcome this situation and decrease the absorption of cholesterol by the body, including the incorporation of phytosterols in foods that can compete with cholesterol and decrease its absorption due to the structural similarity between the cholesterol and phytosterols. Mycosterols have been studied for the same purpose, particularly Ergosterol, which has already been shown to be more effective in reducing the absorption of cholesterol than phytosterols. Thus, the present proposal intended to explore mushroom´s bio-waste as a source of mycosterols, turning this bio-waste in an economic valuable raw material through the extraction of added value molecules. For this purpose, mycosterols were extracted from *Agaricus bisporus* L. by ultrasound-assisted extraction (UAE) in previously optimized conditions, characterized in terms of mycosterols (ergosterol) by HPLC-UV, and further evaluated for their hypocholesterolemic potential in CaCo2 cells. Furthermore, the enriched mycosterol extracts and the free ergosterol were incorporated in cottage cheese, in the free form and their stability and effects on the cheese characteristics were evaluated over the shelf life of the product. From the obtained results, it can be stated that the incorporation of *A. bisporus* extract and pure ergosterol did not cause significant differences in the cottage cheese. Regarding the physicochemical parameters, the color of cheese incorporated with the extract was the brownest compared to the other two samples (cottage cheese with ergosterol and control cottage cheese). Concerning the fatty acids, a dominance of palmitic acid followed by oleic and capric acids was detected. Saturated fatty acids were predominant in the samples followed by mono and polyunsaturated ones. Likewise, two types of sugars have been detected, lactose and glucose. The latter has been observed only in cheese incorporated with *A. bisporus*. In terms of microbial growth, the incorporations did not cause any significant alterations to the normal microorganisms found in the cottage cheese. From the hypocholesterolemic evaluation, it was possible to determine that Caco2 cells absorbed 43.89% of the cholesterol from the control cheese. On the other hand, cheese with pure ergosterol reduced cholesterol absorption by approximately 21.1%, while cheese with *A. bisporus* reduced absorption by approximately 30.24%. These results highlight the capacity of ergosterol to reduce the absorption of cholesterol, being an interesting candidate for the development of functional foods for hypocholesterolemic effects.
Keywords: Cottage cheese, functional foods, mycosterols, hypocholesterolemic agents.

Resumo

Atualmente, os níveis elevados de colesterol são uma preocupação global, devido às doenças graves relacionadas com o aumento deste esterol no organismo. Avanços significativos foram feitos pela comunidade científica para superar essa situação e diminuir a absorção de colesterol pelo organismo, o que incluiu a incorporação de fitoesteróis em alimentos. Estas moléculas competem com o colesterol e diminuem sua absorção devido à sua semelhança estrutural. Embora os fitoesteróis sejam eficientes, novas moléculas têm vindo a ser alvo de estudo para o mesmo objetivo, como os micosteróis, nomeadamente o ergosterol. Este, pode ser extraído de cogumelos e, embora não seja muito explorado, já demonstrou maior eficiência na redução da absorção do colesterol comparativamente com os fitoesteróis. Assim, a presente proposta pretende explorar bio resíduos de cogumelos *Agaricus bisporus* provenientes da indústria, como fonte de micosteróis, transformando-os em matéria-prima de valor económico uma vez que podem ser utilizados para extração de moléculas de elevado valor acrescentado. Assim, bio resíduos de cogumelos foram explorados como fonte de micosteróis, transformando-os em matéria-prima de valor económico pela obtenção de moléculas de valor acrescentado. Os micosteróis foram extraídos de *Agaricus bisporus* L. por extração assistida por ultrassom (EAU), caracterizados em termos de micosteróis (ergosterol) por HPLC-UV, e avaliados quanto ao seu potencial hipocolesterolémico em células CaCo2. Estes extratos e o ergosterol puro foram incorporados em requeijão, sendo a sua estabilidade e os efeitos da incorporação nas características do requeijão, avaliados ao longo do tempo de prateleira. Foi possível afirmar que a incorporação do extrato de *A. bisporus* e ergosterol puro não causou diferenças significativas no requeijão em termos nutricionais e em termos de crescimento microbiano. Em relação aos parâmetros físico-químicos, a cor do queijo incorporado com o extrato foi mais acastanhada em relação às outras duas amostras (ergosterol e controlo). Uma predominância do ácido palmítico seguido dos ácidos oleico e cáprico foram detetadas, sendo que os SFA predominaram. A lactose e glucose foram os dois açúcares encontrados, sendo que este último foi observado apenas em queijos incorporados com *A. bisporus*. Foi possível determinar que o queijo com ergosterol puro reduziu a absorção de colesterol em 21,1%, enquanto o queijo com *A. bisporus* reduziu a absorção em 30,24%. Esses resultados destacam a capacidade do ergosterol em reduzir a absorção de colesterol, sendo um candidato interessante para o desenvolvimento de alimentos hipocolesterolêmicos.

Palavras Chave: Requeijão, alimentos funcionais, micosteróis, agentes hipocolesterolémicos.

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Index

List of figures

List of tables

List of abbreviations

ABCG5	ATP- Banding Casette 5
ABCG8	ATP- Banding Casette 8
ACAT2	Acyl-Coenzyme A: Cholesterol Acyl transferase 2
APC	Aerobic pelate count
ATP	Adenosine Triphosphate
AW	Water activity
CACO2	Human Colorectal Adenocarcinoma Cell Line
CC	Cottage cheese
CCE	Cottage cheese with pur ergosterol
CCEXT	Cottage cheese with A.bisporus extract
CFU	Colony Forming Units
CHD	Coronary Heart Disease
[CI]	Confidence Interval
CIS	The Commonwealth of Independent States
CNIEL	National Interprofessional Centre for Dairy Economy
CPEM	Cottage cheese with pure ergosterol
CPEXTM	sheep cheese with A. bisporus extract
CVD	Cardiovascular Disease
DRBC	Dicloran rosa Bengala Cloronfenicol
EMM	Estimated marginal means
ECACC	European collection of cell culture
EP	Ergosterol Peroxyde
FAME	Fatty Acid Methyl Ester
FAO	Food and Agriculture Organization
FW	Fresh Weight
GC-FID	Gaz Chromatography Coupled with a Flame Ionisation Detection
HPLC-RI	High Performance Liquid Chromatography Coupled with Refraction Index
HPLC-UV	High-Pressure Liquid Chromatography coupled with UV Detector
IFCN	International Farm Comparison Network
ISO	Internatinal Organisation for Standardisation
IT	Incorporation time
LDL-c	Density lipoprotein cholesterol
MRS	Man Rogosa and sharpe
MT	Maturation time
NPC1L1	Nieman Pick Disease C1 Like 1
OECD	Organization for Economic Co-operation and Development
PCA	Plate agar
PDA	Photodiode Array detection
PLP2	Porcine liver primary culture
PW	Peptone water
RNA	Ribonucleic Acid
rpm	Number of turns / minute

RPMI-1640	Roswell Park Memorial Institute Medium
RR	Relative Risk
SFA	Saturated fatty acids
SRB	Sulforhodamine
ST	Student test
TEER	Transepithelial electrilical resistance
TPA	Texture profile analysis
UAE	Ultrasound-assisted extraction
UFLC	Ultra-fast liquid chromatography
UPLC-DAD	High-Performance Liquid Chromatography coupled with Diode-Array Detection.
VLDL	Very Low-Density Lipoprotein Cholesterol
VRBG	Purple red bile lactose agar

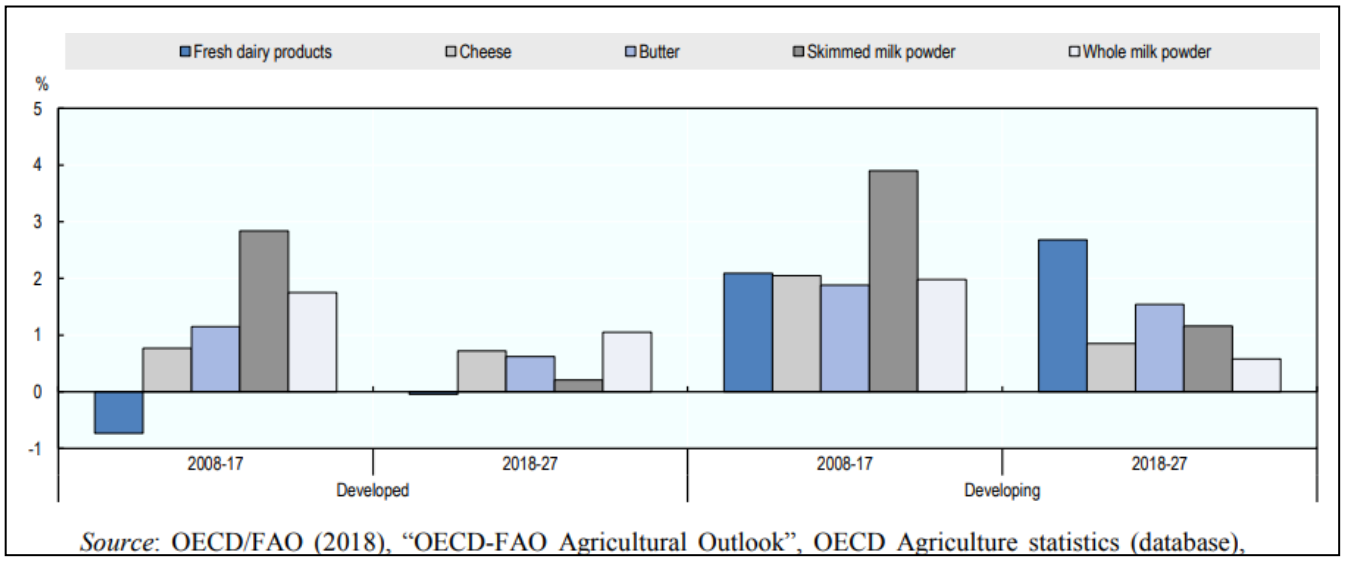
1. Introduction

1.1- Dairy Products and their wide consumption:

There has been a growing interest on functional foods, markedly recognized as being able to provide additional benefits on health promotion, wellbeing maintenance, and disease prevention. Based on this scenario, food industries have been increasingly focused in developing added-value foodstuffs, being dairy products one of the most currently used foods for functional purposes.

1.1.1- Worldwide milk consumption:

World consumption of fresh and processed dairy products is poised to grow by 2.1% p.a. and 1.7% p.a. respectively, over the next decade. The largest share of milk and dairy products consumption is in the form of fresh dairy products, taking up about 50% of the world's total milk production. This share continues to increase to 52% over the next ten years due to rising milk consumption in underdeveloped countries, although consumption dynamics differ considerably between developed and underdeveloped countries. Developed countries consume primarily processed milk products, representing an increasing consumption per capita of 0.7 p.a. (1).



Source: OECD/FAO (2018), "OECD-FAO Agricultural Outlook", OECD Agriculture statistics (database),

Figure 1 : Annual growth rates of dairy products consumption per capita

The method used to calculate per capita consumption is described in the IFCN Dairy Report 2004, which is based on 'milk equivalents' (MEs) so as to account for the consumption of milk in its different forms, such as yoghurts or cheese, in addition to liquid milk (2).

As a general rule milk consumption is high in developed countries and low in the underdeveloped ones, and appears to be particularly low in tropical and subtropical climates. Based on country-specific estimates of per capita milk consumption, the following three categories have been defined (3):

- High, more than 150 kg per capita/year: Argentina, most CIS countries, Costa Rica, Ecuador, Europe, Honduras, Israel, Lebanon, North America, Oceania, Turkey, Uruguay and others such as Pakistan and Sudan.
- Medium, 30-150 kg per capita/year: India, Japan, Republic of Korea, North and Southern Africa, most countries of the Middle East and Latin America (except Argentina, Ecuador and Uruguay).
- Low, less than 30 kg per capita/year: China, Ethiopia, Yemen and most countries of Central Africa and East and Southeast Asia.

1.1.2- Fermented milk products: cheese

Milk products prepared by lactic acid fermentation (yoghurt) or a combination of this and yeast fermentation (Kefir) are usually called fermented or cultured milks.

Fermented milk is the collective name for products such as yoghurt, kefir, cultured buttermilk, cheese and gouta (a Tunisian traditional product). The generic name of fermented milk is derived from the fact that the milk used in the product conception is inoculated with a starter culture which converts part of the lactose in lactic acid. Dependent on the type of lactic acid bacteria, products such as carbon dioxide, acetic acid and several other substances are formed in the conversion process, giving to the final products, a distinctive fresh taste and aroma. Fermented dairy products, particularly cheese, have been used for thousands of years to preserve milk, to make it more transportable, less perishable, readily available, and more digestible, because of lactose breakdown during the fermentation process (4).

It can be hypothesized that this is a highly cultural product both for its production techniques and for its consumption patterns; a product which, by its very diversity, has strong links with production and consumption areas. Nearly 40% of the collected world's milk was used in cheese production in the early 2000s (FAO 2018/OECD). Cheese is also a food at the

forefront of technological innovation and marketing. In addition, this dairy product is the subject of growing international trade. Although in the early 2000s the European intra-Community market accounted for 58% of the world cheese market (Calvez), international flows are less concentrated, indicating that cheese is a food whose production and consumption are growing steadily worldwide.

1.1.2.1- Most consumed cheese and its major consumption countries:

Cheese is mainly consumed in Europe, which accounts for more than half of the world's cheese production. Europe is also the world's largest exporter (ahead of the United States and Oceania), and counts with Japan, Russia and the United States, among the world's leading importers of cheese. It stands out for the great variety of cheeses produced as well as the variety of traditional modes of consumption. Over 2,000 varieties of cheeses exist, and, in France, the two most popular cheeses are Emmental (mainly as a cooking ingredient) and Camembert cheese. Among more than 1500 types of cheese produced in the world, the most purchased and consumed are Camembert, Emmental, Goats, Cheeses, Comté and more surprisingly, the Coulommiers. Cheese consumption, including fresh cheese, averages 17 kg per inhabitant per year in the EU. It can be over 23 kg in France, more than 20 kg in Greece, 22 kg in Germany, 11.6 kg in Canada; and, only 9 kg per person per year in Spain and 11 kg in Poland (CNIEL, FAO, 2016) (5). Although cheese consumption is already high, it is still increasing, also, due to its versatility as a carrier for the dairy industries. The latter themselves promote, through their strategies, the development of worlds production and consumption (6). The figure bellow shows the variation of the cheese consumption in different countries in the world in 2014:

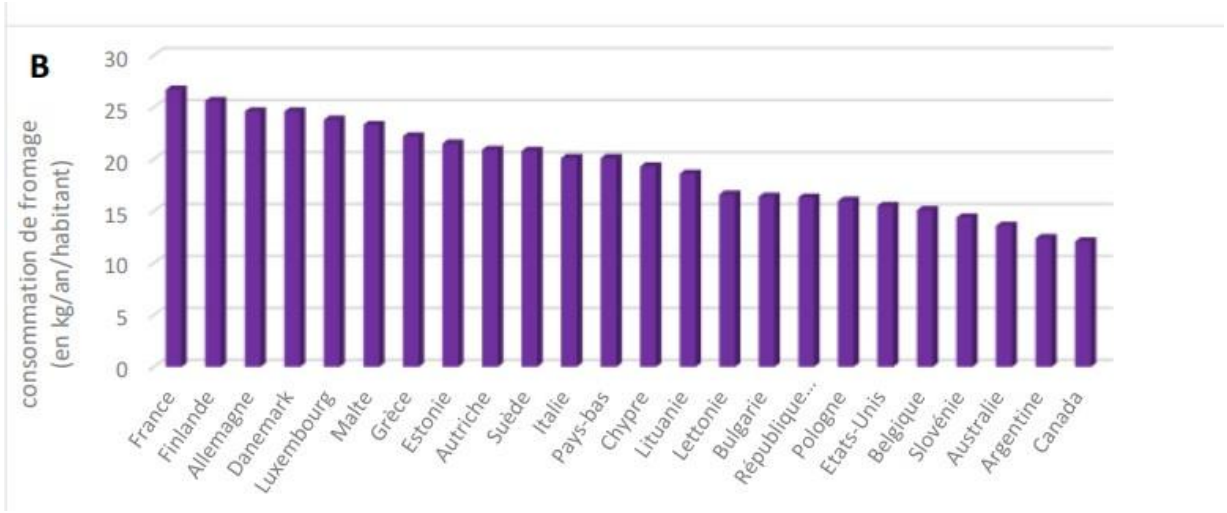


Figure 2: Worldwide cheese consumption in 2014, (7).

1.2- The effect of cheese consumption in health: cholesterol issues

Cheese is a widely consumed, easily digestible, and well tolerated dairy product. It is a rich source of calcium, for the purpose of maintaining bone health, and can also be a good alternative of milk for individuals who are lactose intolerant. On the other hand, cheese also contains a high content of saturated fatty acids (SFA) that could contribute to high low-density lipoprotein cholesterol (LDL-C), a well-defined risk factor for cardiovascular disease (CVD) (8). Dietary guidelines recommend the increasing intake of low-fat dairy and the avoiding of high-fat dairy products as parts of a balanced diet to prevent CVD (9, 10)

The potential cardiovascular effects of cheese have also received great attention over the past two decades, with considerable animal studies and human intervention trials investigating the effects of cheese on CVD risk factors (11–12) along with prospective observational studies that examined whether long-term cheese consumption affects the development of CVD (13, 14, 15). Currently, there are limited certainties achieved. Two recent meta-analyses (16, 17) reported inverse associations of cheese consumption with risks of coronary heart disease (CHD) and/or stroke. The summary relative risk (RR) was 0.84 (95 % confidence interval [CI] 0.71–1.00) for CHD in the analysis carried out by Qin et al. (18), and the summary RR was 0.94 (95 % CI 0.89–1.00) for stroke in the ones described by Hu et al. (19). In addition, potential nonlinear relationship between cheese consumption and risk of CVD remains to be determined. It is relevant to both scientific research and public to understand any threshold effects of cheese consumption on CVD development given the evidence that cheese consumption may be detrimental to certain diseases (e.g., prostate cancer and Parkinson's disease) (20,21).

1.2.1. Phytosterols as hypocholesterolemic agents:

On the terminology base, the term of phytosterols design all the compositions of the same structure deriving from the plants. Phytosterols are chemical homologs to cholesterol and usually found in plants, namely vegetables, especially in the vegetable oils and whole grains. They have a structure similar to that of cholesterol but differ in their C24 side chain and/or the position and configuration of double bonds (Figure 3). Phytostanols are derived from the hydrogenation of phytosterols (22).

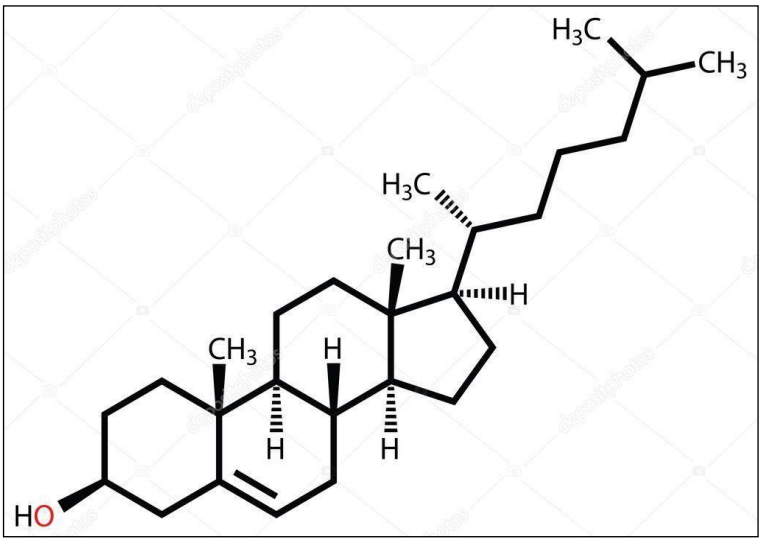
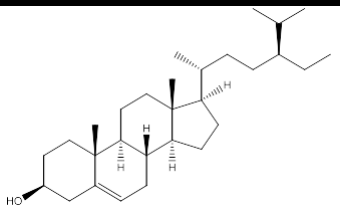
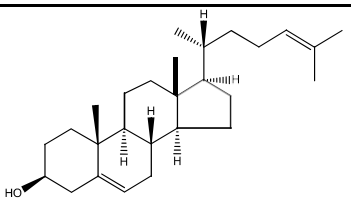
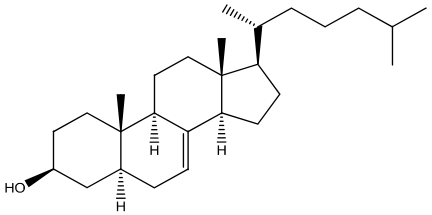
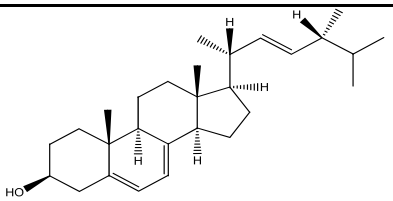
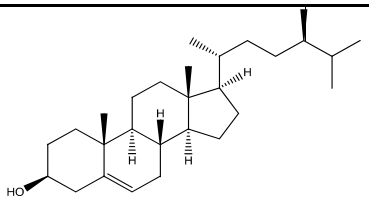


Figure 3: Chemical structure of cholesterol

B-Sitosterol is the main phytosterol found in plants. Moreover, phytosterols and phytostanols are practically identical to cholesterol: in the molecules presented in Table 1, the differences are limited to a double bond, or to a methyl ($-CH_3$) or to an ethyl group ($-CH_2CH_3$), one more or less of the mentioned chemical bound or groups. It can also be noted that phytostanols are the saturated analogues of phytosterols (e.g. comparing sitostanol with sitosterol and campestanol with campesterol). The action of phytosterol and phytostanols on cholesterolemia is due to these strong similarity to cholesterol with small differences (Table 1).

Table 1 : Structures of the main phytosterols and phytostanols of biological importance.

Name	Chemical structure
ß-sitosterol	
Demosterol	
Latosterol	
Ergosterol	
Campesterol	

In fact, several studies have been conducted in order to prove the phytosterols capacity in lowering cholesterol absorption in natural foods (as contrasted with fortified foods). The amount of phytosterols present in natural diets is variable, with reports of between 167 and 437 mg/d in different populations (23, 24, 25). These molecules interfere with the micellar solubilization of cholesterol in the intestine and reduce the efficiency of cholesterol absorption (26), competing with cholesterol for intestinal absorption and thus limiting the absorption of cholesterol.

Phytosterols are less absorbed than cholesterol and their plasma concentration is about 200 times lower than that of cholesterol but it increases with the intake of phytosterols. Phytostanols are even less well absorbed and their plasma concentrations lower than those of phytosterols (22).

The cholesterol-lowering effect of phytosterols has been known since the 1950s. Since then, studies have followed each other in four major phases depending on the doses and forms of phytosterols. Between 1952 and 1976, more than 100 research articles were published discussing the efficacy of phytosterols as cholesterol-lowering agents. These studies included more than 1,800 patients (27). The first of these studies was described by Pollak (28) in 1953, who used sitosterol, the most abundant of phytosterols and verified that with 5 to 7 g/day of sitosterol, they note a 28% decrease in plasma total cholesterol level in patients with high cholesterol levels. In the 1970s, the applied dose of sitosterol was reduced and, as a result from these studies, it is deduced that the ideal dose of sitosterols that can interfere with the intestinal absorption of cholesterol is 3 g/day (29).

1.2.1.1- Bioavailability and mechanisms of action of phytosterols

Bioavailability of phytosterols is very low, due to their crystalline structure and poor water solubility, limiting their potential health benefits.

In the intestinal lumen, before being absorbed, cholesterol is in solution with other fatty substances. Among these fats, monoglycerides and fatty acids are rapidly absorbed by the duodenum while the concentrations of much more difficult to absorb substances, such as sterols, increase. When their concentrations reach a critical threshold, they form crystals which can no longer be absorbed by the duodenum.

Due to their similar structure, cholesterol and phytosterols in the free form are not very soluble in fats and micelles. In addition, when they are present, they hinder each other's respective solubility. As a result, a significant increase in the amount of phytosterols leads to a decrease in the solubility of cholesterol and causes an increase in its precipitation and fecal excretion.

The digestive absorption of cholesterol is carried out from micelles which interact with a specific receptor for the absorption of sterols NPC1L1 (Niemann-Pick C1 Like-1 protein). This specific transporter can be inhibited by ezetimibe (Ezetimibe is a drug used to treat high cholesterol and works by decreasing the absorption of cholesterol ingested from the digestive tract) (30). The digestive absorption of sterols is not only regulated by the influx but also by

the efflux. This latter efflux mechanism is dependent on an ABC transporter (ATP binding cassette transporter protein) which is responsible for the excretion (efflux) of sterols from the enterocyte to the digestive lumen. This influx/efflux system allows regulation according to the intakes and explains that there is a reduced absorption in the event of significant cholesterol intake.

Phytosterols are actively excreted by two ABC transporters, G5 and G8, which use ATP as energy to transport many substances across the membranes. Structurally ABC, G5 and G8 are hemi-transporters that work together like heterodimers. In the enterocyte, cholesterol is preferably esterified by ACAT (AcylCoA: cholesterol transferase) before being incorporated into chylomicrons (Chylomicrons are lipoproteins that are formed during digestion. They are responsible for the transport of exogenous lipids from the small intestine to the peripheral fatty tissues where they are reprocessed). In contrast, phytosterols have less affinity for ACAT and they are then excreted by ABC carriers.

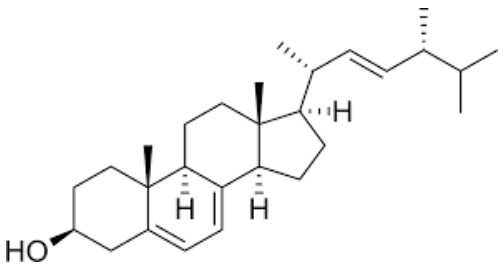
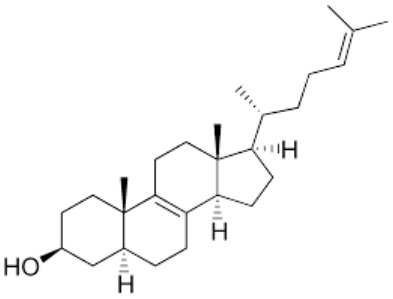
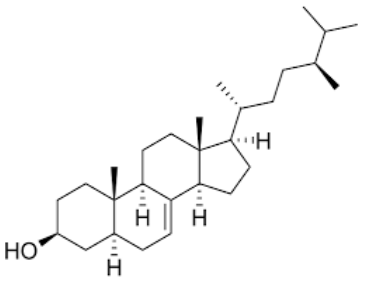
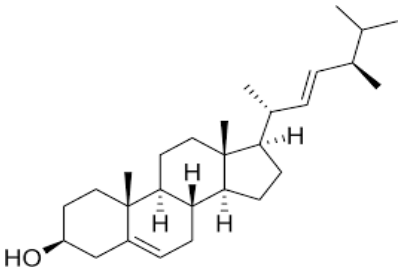
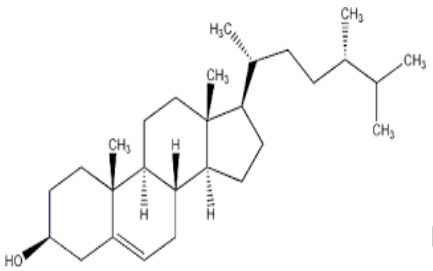
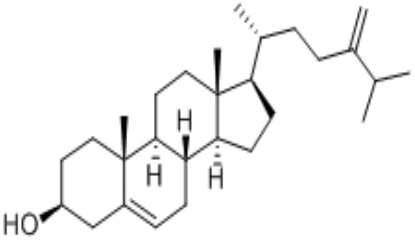
It appears that the ABC G5 and G8 transporters located in the liver also play an important role in the excretion of phytosterols and therefore in regulating their levels in the body (31). Finally, inhibiting the absorption of cholesterol decreases the flow of digestive cholesterol to the liver.

The decrease in the amount of cholesterol in the hepatocyte is not fully compensated by an increase in the synthesis of hepatic cholesterol (reflected by an increase in the activity of 3-hydroxy-3-methylglutaryl-coenzyme A - HMG- CoA - reductase and an increase in circulating lathosterol) and also stimulates the synthesis of LDL receptors, a process which leads to the effective decrease in circulating LDLcholesterol levels.

1.2.1.2- Chemical structures and biosynthetic pathway of mycosterols

Sterols are special forms of steroids that can be found in animals (zoosteroids), plants (phytosteroids) and fungi (mycosteroids) (32). They play an important physiological role to the extent that they are vital compounds of the plasma membrane, being also responsible for maintaining the structure and viability of cells. Ergosterol (1) is the main sterol in fungi, but Zymosterol (2) fungisterol (3), ergosta-5,7-dienol (4), 24-methyl cholesterol (5), and methylene cholesterol (6) (table 2) are further examples of important sterols in fungi (33, 34).

Table 2 : Chemical structures of common mycosterols

 (1) ergosterol	 (2) zymosterol	 (3) fungisterol
 (4) ergosta-5,7-dienol	 (5) 24-methyl cholesterol	 (6) methylene cholesterol

The synthetic pathway leading to fungal sterols, which involves at least 20 enzymes, uses an isoprenoid pathway to produce squalene oxide. This pathway is very similar to that one involved in cholesterol formation (35). Most of the mycosterols are distinguished by the methylation of lanosterol at C-24, and subsequently follow a series of demethylations at C-4 and C-14 and double bond transformations that (in most cases) produce the C28 sterols that are frequent in most of the fungi (36). Diverse pathways culminating with the formation of the C28 sterol ergosterol vary in accordance with the sequence of double bond transformations (37). However, in some taxa, a second methylation that originates a 24-ethylidene, which is latter reduced to 24-ethyl, produces C29 sterols (36). Most of these sterols are intermediates in the biosynthesis of ergosterol (Figure 4).

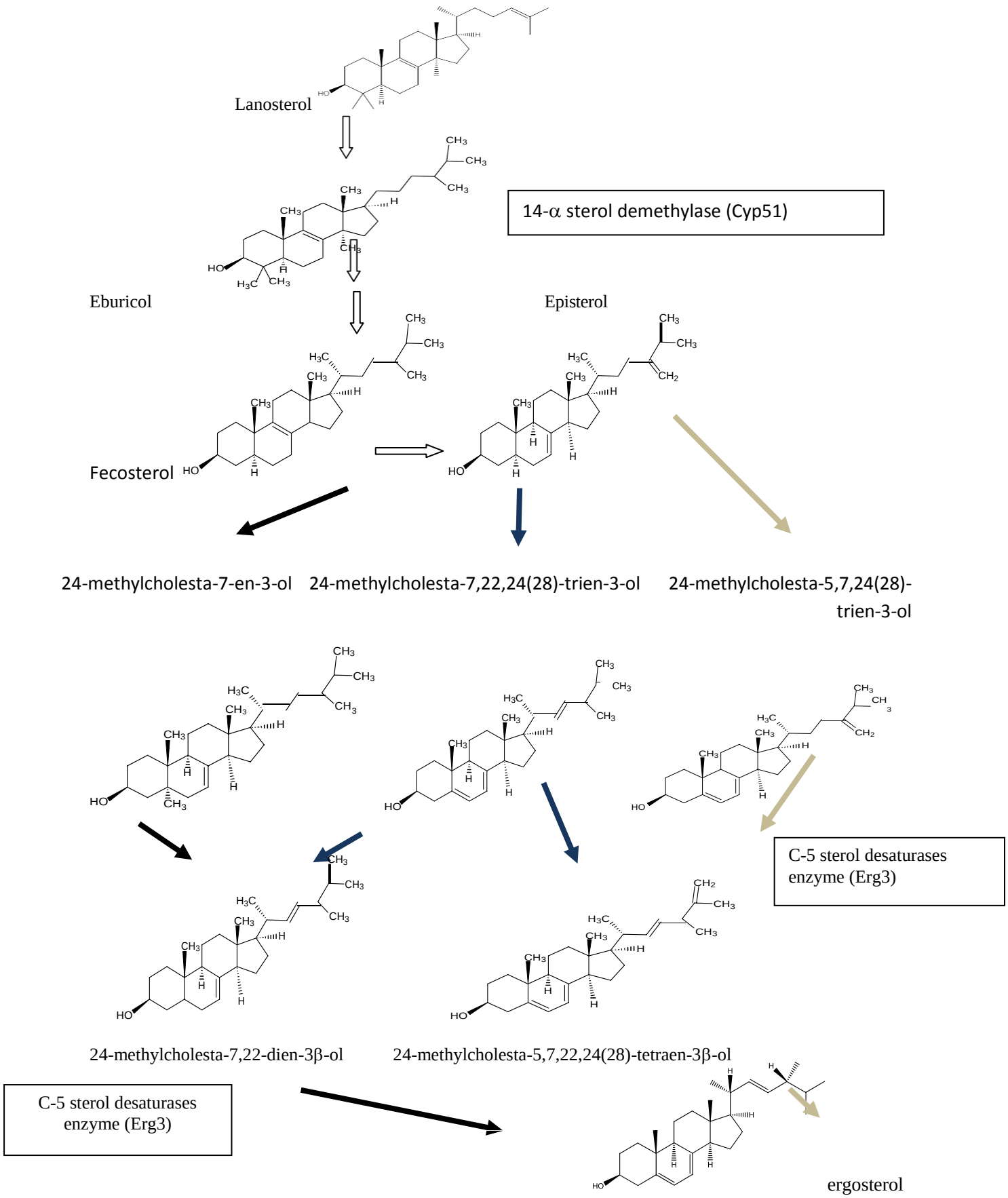


Figure 4: Biosynthesis of ergosterol (38)

1.2.2. Hypocholesterolemic potential of Mushrooms (mycosterols)

From the health benefits point of view, ergosterol has great importance, not only due to its own biological properties, but also because it can be converted into vitamin D2 after photolysis and thermal rearrangement (39). Moreover, ergosterol was reported to be effective as anti-inflammatory, antitumor, antibacterial, antioxidant, antiviral and also hypocholesterolemic agents.

The mechanisms related to cholesterol metabolism involved in the hypocholesterolemic effect of edible mushrooms are depicted in Figure 5. The fatty acid pattern of edible mushrooms seems to contribute to reduce serum cholesterol levels (40,41). When the fatty acids profile of some edible mushrooms was analyzed, considerable amounts of polyunsaturated fatty acids were found. The soluble dietary fiber has also shown healthy effects on serum lipid levels, reducing total cholesterol and LDL-cholesterol amounts (42). The formation of viscous gels from soluble dietary fiber such as glucans might contribute to inhibit the cholesterol and triglycerol absorption (43). Their viscous properties are related to an increase on the fecal excretion of bile acids and short-chain fatty acids (propionate), which inhibits acetate incorporation (substrate for sterols and fatty acid synthesis) to serum lipids. The results of some reports suggest that the hypocholesterolemic effects of some fruiting bodies of edible mushrooms could be mainly attributed to the dietary fiber supply.

Furthermore, as phytosterols, there is a competition between cholesterol and ergosterol for the incorporation of dietary mixed micelles which will reduce the amount of the cholesterol absorbed by the enterocytes

Ergosterol was pointed as a potent agonist for liver X receptor (a factor involved in the inhibition of the cholesterol absorption by up-regulating ABCGbcg5 and ABCGbcg8 genes in the small intestine) and as an inducer of ABC-transporters expression (promoters of the active efflux of cholesterol and plant sterols from the enterocyte into the intestinal lumen for excretion). It was also described as a potent C24-reductase inhibitor, an enzyme which catalyzes the reduction of the double bond at C-24 in the cholesterol-biosynthesis pathway.

It can also combine with niacin to inhibit the activity of the enzyme hydroxymethylglutaryl-CoA reductase; which controls the synthesis of cholesterol by the liver, and, as a result, the amount of endogenous cholesterol will decrease.

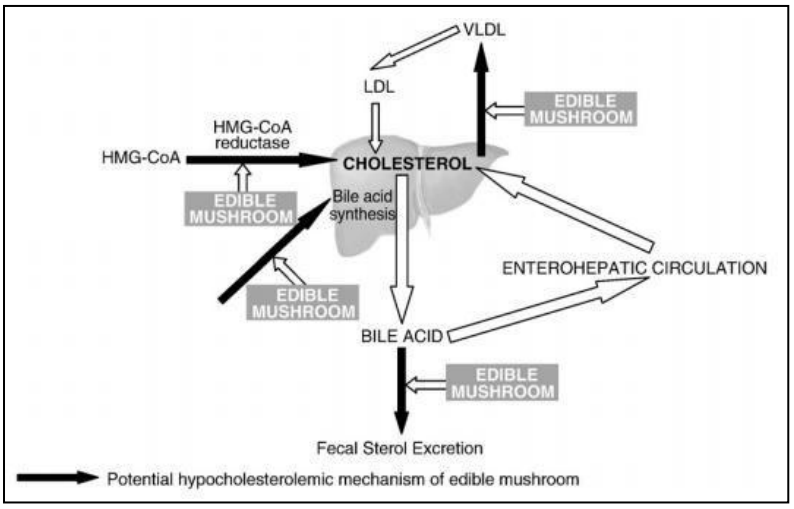


Figure 5: Effect of edible mushrooms on the cholesterol metabolism

1.3. Dairy products functionalized with hypocholesterolemic agents

In the last decades consumer demands in the field of food production has changed considerably. Consumers more and more believe that foods contribute directly to their health (45). Today foods are not intended to only satisfy hunger and to provide necessary nutrients for humans but also to prevent nutrition-related diseases and improve physical and mental well-being of the consumers (46). The role of food as an agent for improving health has proposed a new class of food, called functional food. Typically, a food marketed as functional contains added, technologically developed ingredients with a specific health benefit (47).

Milk and dairy products have been associated with health benefits for many years containing bioactive peptides, probiotic bacteria, antioxidants, vitamins among others.

Consumer's increasing interest for maintaining or improving their health by eating these specific food products has led to the development of many new functional dairy products. These dairy products may contain many functional ingredients that have been added (ergosterol) and that decrease the absorption of cholesterol, can significantly reduce blood pressure, play role in the regulation of satiety, food intake and obesity-related metabolic disorders and may exert antimicrobial effects.

1.3.1. Efficacy (Incorporation, doses and intake)

The consumption of foods enriched with phytosterols (PS) can promote substantial reductions (8 and 10%) in the low-density lipoprotein cholesterol (LDL-C) concentrations, a major risk factor for CVD (48).

Dietary intake of phytosterols ranges from 150-400 mg/day in a typical western diet. Phytosterols and phytostanols, in free or esterified form, are added to foods for their properties to reduce absorption of cholesterol in the gut and thereby lower blood cholesterol

levels. It is now generally accepted that sterols and stanols have the same cholesterol lowering efficacy (49). The daily doses, considered optimal for the purpose of lowering blood cholesterol levels, are 2-3 g of phytostanols and/or phytosterols, which translates to 3.4-5.2 g in esterified form. This recommended daily dose is typically divided in 1-3 portions of food providing 1.7-5.2 g ester, which equals 1-3 g phytostanol and/or phytosterol equivalents (49)

However, some studies have already examined the phytosterol supplementation's hypocholesterolemic effects in low-fat dairy products such as milk, yogurt, and other beverages. Indeed, an absolute decrease in plasma cholesterol concentration has been observed.

Also, some studies have shown that a daily intake of phytosterols, by people with moderate hypercholesterolemia decreases blood cholesterol levels from the 3rd week and remain present after 6 weeks of active consumption of phytosterols (50).

The variety of food matrices used in phytosterol functional foods has expanded beyond margarine spreads as incorporation techniques for esterified and free phytosterols have improved. Mayonnaise, salad dressings, dairy and nondairy beverages, chocolates, meat, cheese, and baked goods have all been used to deliver phytosterols in clinical trials (51). Clifton et al. (52). Compared the LDL cholesterol lowering effects of 1.6 g/day of phytosterols delivered by milk, yogurt, bread, or cereal. The LDL cholesterol lowering action of the phytosterol enriched milk was 15.9%, almost twice that of the yogurt (8.6%) and three times that of bread and cereal (6.5 and 5.4%, respectively).

This was the first study to show conclusively using a head to head comparison that food matrix can significantly affect LDL cholesterol lowering of phytosterols. The effect of food matrices on LDL cholesterol lowering of phytosterol supplementation has since been investigated in a meta-analysis by Abumweis et al. (51) who concluded that phytosterols incorporated into fat spreads, mayonnaise, salad dressing, or dairy reduced LDL levels to a greater extent than incorporated into other products such as baked goods and juices. Demonty et al. (53) also reviewed the effect of phytosterol food matrix on cholesterol lowering, high fat to low fat foods and dairy to non-dairy foods were compared and no differences in the absolute or relative dose response curves of the different food formats was seen. These meta-analyses findings of Demonty et al. (53) and Abumweis et al. (51) are not contradictory because classification of food formats compared differed between analyses.

In a recent study, it has been proven that yogurts incorporated with Ergosterol show higher antioxidant, antitumor and anti-inflammatory activities than the commercial samples incorporated with phytosterols; being the Ergosterol content in the yogurts much lower than

the ones with phytosterols, highlighting the stronger activity obtained with lower amounts of Ergosterol.

In the European Union, the cheese consumption is around 10 million tons per year, averaging a consumption of about 14.2 kg in 2017 per capita. The top cheese consumer is Denmark, with an average consumption of 28.1 kg of cheese, followed by Iceland with 27.7 kg, Finland at 27.3 kg and France with 27.2 kg. The high-fat dairy products are known to increase high density lipoprotein (HDL) and low-density lipoprotein (LDL)-cholesterol concentrations which predicts risk of cardiovascular diseases. Analyses conducted in Iran on 1.752 participants (782 men and 970 women) demonstrated that cheese eaters had higher levels of C-reactive protein, apo-lipoprotein A, HDL cholesterol, while fasting blood pressure, total cholesterol, LDL cholesterol, Apo B and triglyceride where not any higher. Considering all this evidence, cheese can be an outstanding candidate for incorporation of Mycosterols or extracts containing this molecule.

1.4. Extraction methodologies for Mycosterols

Besides the solid-liquid extraction methods mostly applied in mycosterols, there are the conventional: maceration and Soxhlet extractions; and emerging: ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid and accelerated solvent extraction. These new techniques have been developed in order to decrease the extraction time and the solvent consumption, so that enhancing the extracts quality. (53)

Table 3: Advantages and disadvantages of techniques applied to the mycosterols extraction

Technique	Advantages	Disadvantages
Maceration	Simple and inexpensive	High extraction times
Soxhlet	Standard method/ Low costs/ Uses large amount of samples/ No filtration	Slow method Large amount of solvent
Ultrasound	Rapid technique/ Low costs/ Large amount of samples	Large amount of solvent Filtration
Supercritical Fluid	Rapid/ Selective method when varying pressure and temperature/ Requires small amount of solvent and can be fully automated/	Use of high pressures High costs
Microwave	Rapid technique/ small amount of solvent compared with soxhlet/ Complete control of extraction parameters	Requires Filtration
Accelerated solvent	Rapid technique/ Minimal amount of solvent/ fully automated and easy to operate	Very high cost

1.4.1. Ultrasound-assisted extraction (UAE)

The ultrasound technique is a process that uses the energy of the waves (mechanical) transmitted in frequency higher than 20 kHz. This energy is used in order to convert electrical energy into vibrational impulses (55).

There are two commons devices for ultrasounds extraction. The more widely used is the bath system but the one which offers more advantages is the probe system. This provides directly cavitation in the solution, being more efficient (56).

The main benefits of using ultrasound in solid–liquid extraction include the increase of extraction yield and faster kinetics.

Ultrasound can also reduce the operating temperature allowing the extraction of thermolabile compounds. It is necessary to consider sample characteristics such as moisture content, particle size, solvent used for the extraction in order to obtain an efficient and effective ultrasound-assisted extraction. Furthermore, many factors govern the action of ultrasound including frequency, pressure, temperature and sonication time (54). The UAE has been used as an alternative to extraction by Soxhlet (54), being methanol, dichloromethane and chloroform the most used solvents in this technique in a ratio that can increase the extraction efficiency of sterols. This extraction technique is the most suitable for the extraction of mycosterols when compared for instance with the soxhlet (Heleno et al., 2016), (57) since the UAE can provide the same amount of extract and the same purity, with significantly reduced time and solvent.

1.5. Objectives:

This proposal aims at incorporating *Agaricus bisporus* L. extracts which contains mycosterols, and pure Ergosterol in sheep cottage cheese, in order to develop a functional food with hypocholesterolemic effects.

The principal objectives are:

i) Extracting Mycosterols from *A. bisporus*;

ii) Evaluating the hypocholesterolemic activity of the obtained extracts and pure ergosterol;

iii) Incorporating *A. bisporus* extracts and pure ergosterol in cottage cheese;

iv) Evaluating the nutritional value, physicochemical parameters (color, pH, texture and water activity), individual composition in fatty acids and microbial load in the final formulations;

v) Evaluating the stability of the ergosterol molecule over the shelf life of the cottage cheese.

1.6. Methodology

1- Extraction of mycosterols by the ultrasound assisted extraction (UAE) technique;

2- Identification and quantification of mycosterols (ergosterol) by HPLC-UV;

3- Evaluation of the hypocholesterolemic activity using a CaCo2 cell line;

4- Incorporation of the extracts and the pure ergosterol in sheep cottage cheese in the Queijaria vaz enterprise;

5- Evaluation of the nutritional value of the developed cheeses, according to the AOAC (2016) procedures;

6- Evaluation of the physical parameters of the developed cheeses, namely texture (TA.XT Plus Texture Analyser) assessing hardness, cohesiveness, adhesiveness, chewability and elasticity; colour using a colourimeter (CR400 da Konica Minolta (Chiyoda, Tokyo, Japan) with illuminant D65 using the L*, a* and b* measurement standards, where L* represents the brightness and a* and b* the color space, ranging from red-green to yellow-blue; and water activity (a_w) by using an activity meter instrument (AQUALAB 4TE) based on the dew-point method;

7- Evaluation of the microbial load over the product shelf life according to the *International Organization for Standardization* (ISO) 6887-1:2003;

2. Material and methods

2.1. Mushroom samples

2.1.1. *Agaricus bisporus L.*

The samples of *Agaricus Bisporus* L. were provided by a local mushooms enterprise "Mogaricus-Sociedade UnipessoalLda", and consisted of discarded bioresidues, namely mushrooms that do not meet the marketing requirements such as samples with broken shape, and misshapen physiognomy. This raw material was frozen at -30 ° C and lyophilized (FreeZone 4.5 model 7750031, Labconco, KS, USA). The dried samples were reduced to a dried fine powder (20 mesh) (figure 6)[1].

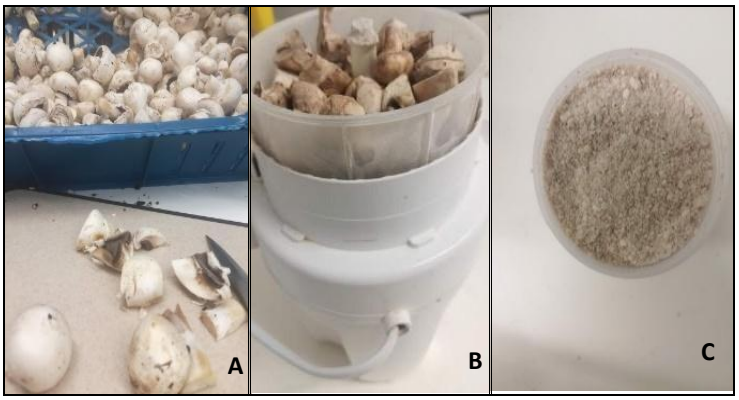


Figure 6 : Mushroom preparation: A-Lyophilized mushroom, B-mushroom grinding, C-mushroom powder

2.2. Extraction procedures

2.2.1. Preparation of the mycosterol enriched extracts

The mycosterol extracts were obtained by ultrasound assisted extraction (UAE) as previously developed by Heleno et al.(57). 3 g of lyophilized mushroom powder were extracted with 100 ml of ethanol in the UAE (ultrasonic homogenizer 'CY-500') system for 15 min at a power of 375 w. After that, the extract solution was filtered through a whatman paper number 4 and the obtained solvent was evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) (Figure 7).

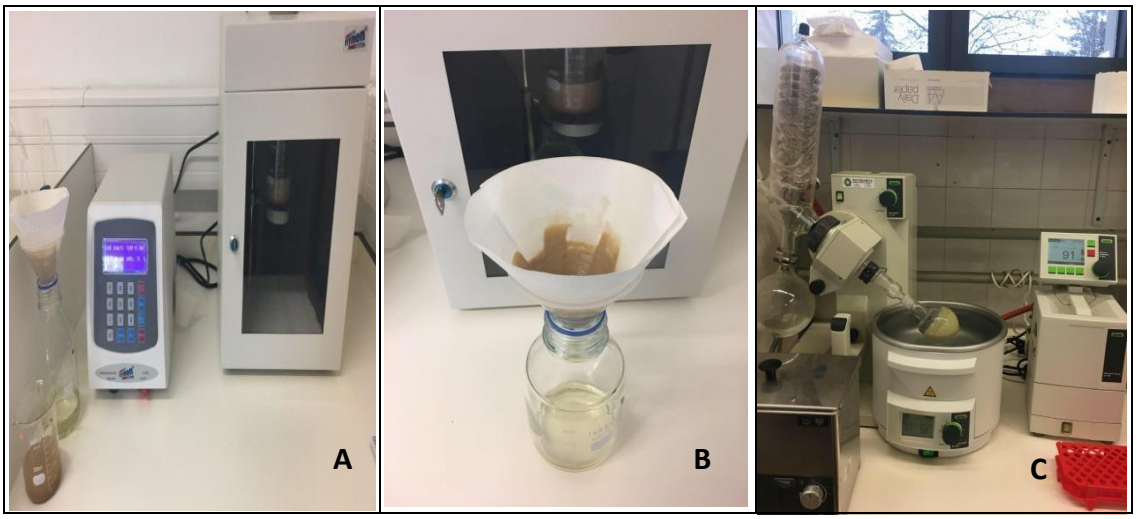


Figure 7: Obtention of ergosterol extracts: A- UAE extraction, B- Filtration, C- Solvent evaporation

2.2.2. Obtention of the cheese extracts

For the extraction of the cheese samples, specifically for ergosterol and cholesterol quantification, the soxhlet extraction technique was selected based on the higher capacity to extract higher amounts of these compounds from the cheese samples (Heleno et al., 2016).

About 3g of cheese powder was extracted with 150ml of ethanol over 5 hours (14 cycles) and refluxed in the soxhlet apparatus. The solvent was then evaporated under reduced pressure (Buchi R-210 rotary evaporator, Flawil, Switzerland).

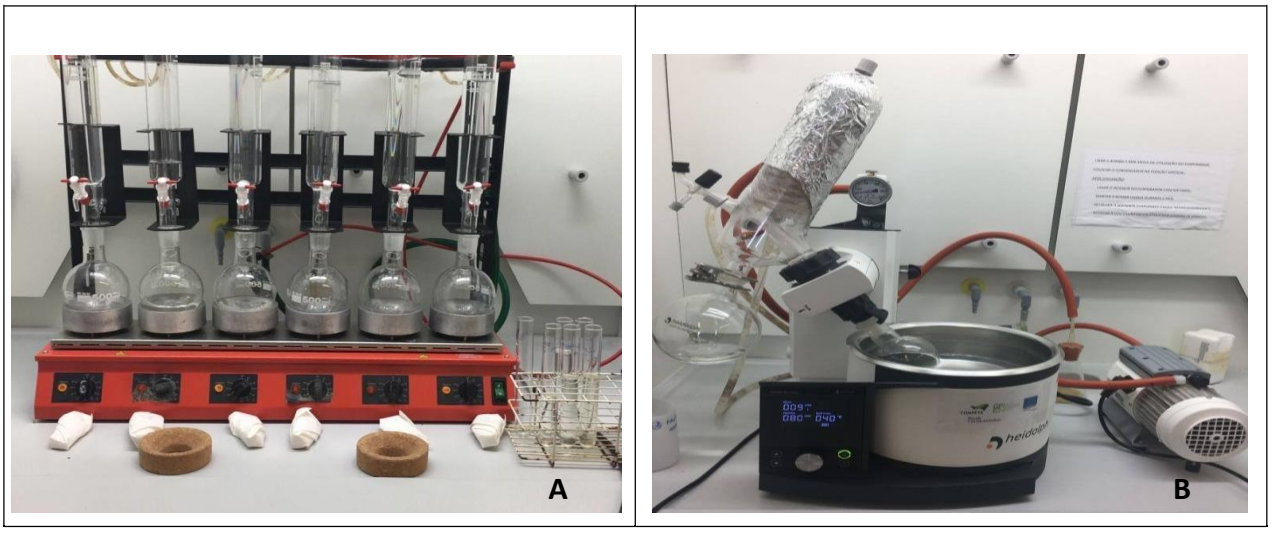


Figure 8: Soxhlet extraction; A-extraction, B-solvent evaporation

2.3. Incorporation of *A. bisporus* extract and pure ergosterol in cottage cheese

2.3.1. Cottage cheese making process

The cottage cheese was made at the artisanal manufacturing plant of the enterprise "Queijaria Vaz" located in Mirandela, Portugal. For its production, after the milk collection, it was warmed at 30ºC for coagulation (4-5 h). After the milk was clotted, a lateral tap was opened for the removal of the whey. This whey is placed in a container and warmed at 83-85ºC. During this process, floccules are formed and manually removed and placed in the cottage cheese shapes with tiny holes. These shapes with the floccules are left to stand about 24h, so that the excess of whey can get out. After this period, the cottage cheese is packed in a plastic container and kept in a refrigerated room at 4-5 ºC. (Figure 9)

Figure 9: Cottage cheese

2.3.2. Incorporation process

The incorporation contents were based on the amount of ergosterol, taking into account the amount of similar molecules already used as hypocholesterolemic agents, the phytosterols (80 mg of phytosterol´s extract per g of margarine) (Law, 2000). (58)

Thus, two different cottage cheeses were prepared: i) 40 mg of *A. bisporus* extract/g of cottage cheese because de amount similar to the one of phytosterols incorporations caused significant rheological changes in the cottage cheese, so the doses was decreased to half; ii) 3 mg of pure ergosterol per g of cottage cheese, corresponding to the ergosterol content in 80 mg of *A. bisporus* extract, the initial planned incorporation amount.

The different formulations were incorporated manually as follows: the cottage cheeses were removed from the shapes, homogenized with two formulations, in triplicate and further inserted in the corresponding shapes, obtaining the final formulations: Cottage cheese with *A. bisporus* extract (CCEXT) (Figure 2A) and cottage cheese with pure ergosterol (CCE) (Figure 2B), and cottage cheese without any of the selected ingredients (CC).

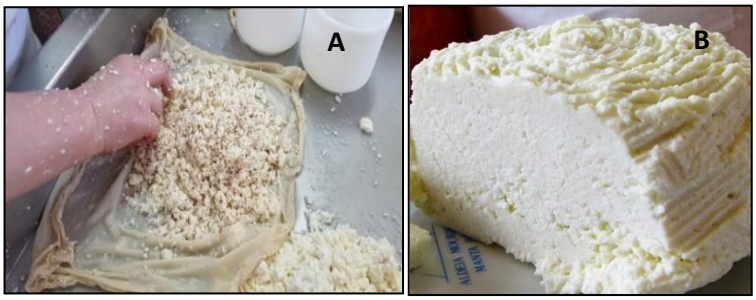


Figure 10: Incorporation process

2.4. Chemical composition

2.4.1. Nutritional value

Moisture, fat, protein and ash was determined following the standard AOAC procedures (AOAC, 2016). (59)

For the fat, a crude was determined by extracting a known weight (1g) of powdered sample with petroleum ether, using a Soxhlet apparatus; The crude protein content (N×6.38) of the samples was estimated by the macro-Kjeldahl method and the ash content was determined by incineration at 600 ± 15 °C.

2.4.1.1. Moisture

The samples (2g) were placed in the balance moisture analyzer (Adam Equipment, PMB 163). This equipment slowly raises the temperature to 105°C to force moisture to evaporate from the food. When the weight is constant and no evaporation is registered, the sample is weighed again. The results were obtained using the following function:

.

With is the initial weight and is the weight after reaching a constant weight.

Figure 11: Moisture analyzer

2.4.1.2. Fat

Fat content was determined by extracting 1g of each sample with petroleum ether in a soxhlet apparatus. After that, and to evaporate the petroleum ether, the solution was placed in the oven and the crude fat was then weighted apart.

Figure 12: Fat extraction

2.4.1.3. Proteins

The protein analysis was conducted using the M.Kjeldahl process, which quantifies the raw protein content as a function of the nitrogen content of the samples. For that, 250mg of samples were weighted and introduced with two selenium pellets (as digestion catalysts) and 15 mL of concentrated sulfuric acid into the digestion tube.

Thereafter, the tubes were placed in the digester (FossTM Digestor) for 70 min at 400°C. After cooling, the tubes were placed in the Kjeldahl apparatus, which automatically insures distillation and titration. The protein content was then calculated by multiplying the value obtained for nitrogen by a conversion factor selected on the apparatus (N×6.38).

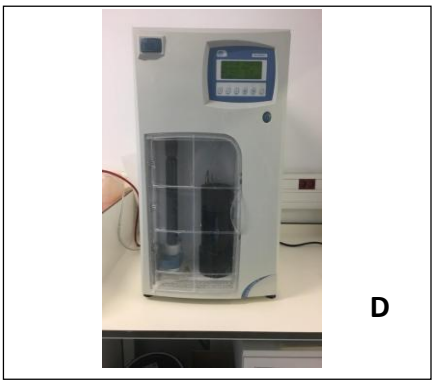


Figure 13: protein analysis; A-Addition of selenium pellets, B-Digestion, C- Digested sample, D-Titration

2.4.1.4. Ash

For this analysis, 250mg of cheese sample were weighed in a crucible and placed overnight in a muffle furnace at 600°C ± 15°C for incineration. The results were expressed as a percentage of total ash following the equation:

% — .

Where the initial mass represented by and the final mass by , Corresponding to the residues after incineration.

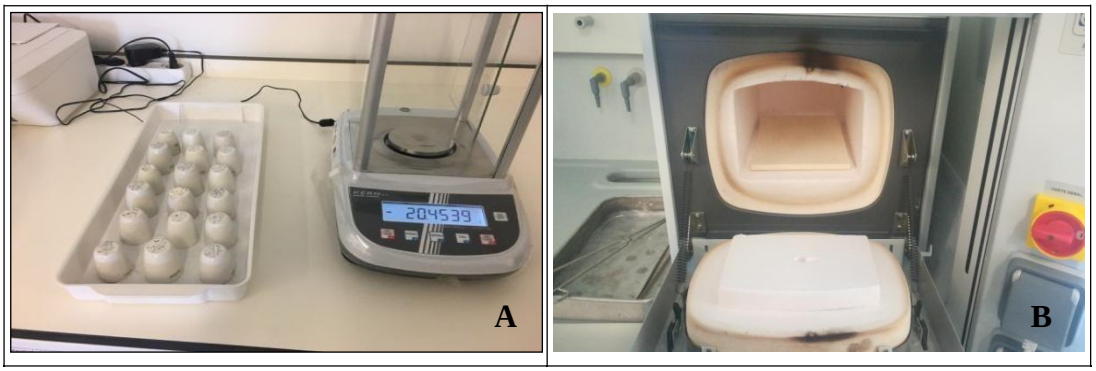


Figure 14: A- Sample preparation; B-Muffle furnace for incineration

2.4.2. Fatty acids

After obtaining the lipid fraction by Soxhlet extraction, fatty acids were determined according to a transesterification process while using 5mL of methanol: sulfuric acid: toluene 2: 1: 1 (v: v: v:), for 12h at least in a water bath at 50°C, 60rpm.

After esterification, 3 ml of distilled water was added to obtain two distinct phases. The fatty acid methyl esters (FAME) were recovered with 3 ml of diethyl ether, vortexed and finally the hydrophobic phase was collected in flasks containing anhydrous sodium sulfate in

order to remove the existing traces of water. Subsequently, the samples were filtered using a 0.2 µm nylon filter (Whatman) and analyzed by gas chromatography (DANI 1000, Contone, Switzerland) coupled with a flame ionization detection (GC-FID)/capillary column.

Analysis was performed with a split / splitless injector, an FID at 260 ° C and a Zebron-Kame column (30m x 0.25mm id x 0.20 µm film thickness, Phenomenex, Torrance, CA , USA). The carrier gas flow rate (hydrogen) was 1.1 mL / min, measured at 100 ºC. A fractional injection (1:50) was performed at 250 ºC.

Fatty acids identification and quantification was performed by comparing the relative retention times of fatty acid methyl ester peaks with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed as a relative percentage for each fatty acid as described by Pinela et al., 2011.(60)

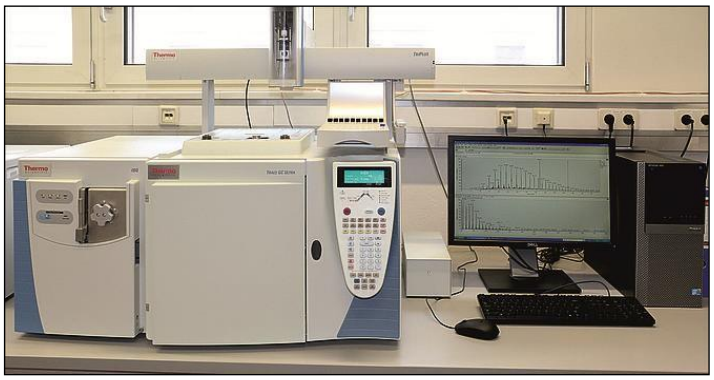


Figure 15: GC-FID

2.4.3. Sugars

For the sugar determination, 0.5 g of the sample was mixed with raffinose (internal standard, IS, 25 mg / ml) and with 40 ml of 80% aqueous ethanol at 80°C for 1 h 30 min, with shaking every 15min (to let out the gazes). The resulting suspension was then centrifuged (Centorion K24OR-2003 refrigerated centrifuge) at 15,000 g for 10 min and then transferred to a 250ml ground glass flask. The supernatant was filtered, and subsequent evaporation of the solvent and removal of excess fat was carried out by washing the suspension 3 x 5 ml of ethyl ether. After that, the aqueous phase was put into a tube and left overnight in the oven to evaporate the rest of ether, make up the extract with 5ml of water, filter to a 10ml vial, transfer 1.5ml of sample to a vial and analyze by HPLC.

HPLC consisted of an integrated system with a pump (Knauer, Smartline system 1000), auto-sampler (AS-2057 Jasco) and degasser system (Smartline manager 5000) coupled

to a refraction index detector (RI detector Knauer Smartline 2300) showed in **Figure 16** as previously described by the authors Pinela et al., 2011. The chromatographic separation was achieved with a Eurospher 100-5 NH_2column (4.6 × 250 mm, 5 µm, Knauer) operating at 30 °C (7971 R Graceoven). The mobile phase was acetonitrile/deionized water (70:30, *v*/*v*) at a flow rate of 1 mL/min.

Lactose was identified by comparing the relative retention time of sample peaks with a standard. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of the standard, using the internal standard method and by using calibration curves obtained from a commercial standard. The results were expressed in g per 100 g of fresh weight.

Figure 16: HPLC-RI

2.4.4. Organic acids

For the organic acids, 1 g of the sample was mixed with 25 ml of metaphosphoric acid (4.5%) into a goblet covered with aluminum foil. The mixture was then placed under magnetic stirring for maceration for 20 min at the room temperature. After this process, the suspension was filtered through a 0.2µm nylon filter (Whatman)

The lactic acid was determined by ultra-fast liquid chromatography UFLC Shimadzu 20A series (Shimadzu Corporation, Kyoto, Japans) coupled to a photodiode array detector (PDA). A SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 µm, 250 mm × 4.6 mm i.d internal diameter.) thermostatted at 35 ºC was used. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out using 215 and 245 nm (for ascorbic acid) as preferred wavelengths. For the quantitative analysis, a calibration curve with known concentrations of a commercial standard

was constructed, and the lactic acid present in the two samples was determined by peak area comparison at 215.

Figure 17: UPLC-DAD

2.4.5. Ergosterol and cholesterol quantification

The obtained mushroom extracts by UAE and the cheese extracts obtained by Soxhlet extraction were dissolved in methanol in order to obtain a concentration of 20 mg/mL and filtered through a 0.2 μm nylon filter for ergosterol and cholesterol quantification by HPLC-UV according to Heleno et al., 2016. (57)

The equipment consisted of the same Knauer system described above. Chromatographic separation was achieved with an Inertsil 100A ODS-3 reversed-phase column (4.6 × 150 mm, 5 μm, BGB Analytik AG, Boeckten, Switzerland) operating at 35 °C (7971R Grace oven).

The mobile phase was acetonitrile/methanol (70:30, v/v) at a flow rate of 1 mL min^{-1}, the injection volume was 20 μL and the detection was performed at 200 nm for cholesterol and 280 nm for ergosterol.

The quantification was achieved based on calibration curves obtained from commercial standards using the internal standard method with cholecalciferol as the internal standard. Data were analyzed using Clarity 2.4 Software (DataApex). (Table 4)

2.5. Physical parameters

2.5.1. Color

The external color was analyzed for each cheese sample. This analysis was performed with a portable CR400 colorimeter from Konica Minolta (Chiyoda, Tokyo, Japan) with the D_{65} illuminant, a standard illuminant defined by the International Commission on Illumination (CIE) which represents the midday light in Europe (daylight illuminant).

The CIE L* a* b* color space of 1976 was used, with L* representing lightness, a* representing redness (red-green), and b* representing yellowness (yellow-blue), with a 10º observer angle and 8 mm aperture.

Figure 18: Colorimeter

2.5.2. Texture

Texture analysis was carried out on a Stable Micro Systems (Vienna Court, Godalming 191 UK) TA.XT Plus Texture Analyzer with a 30 Kg load cell, using the P/45 45mm aluminium cylinder probe (**Figure 19**) according to Carocho et al., 2019. **(61)**

A pre-test speed of 5mm/s, a post-test speed of 5mm/s and a test speed of 3mm/s were used to evaluate the texture profile analysis (TPA) on the samples. The goal mode was set to strain 5% of the samples of cheese that were compressed twice for 5 seconds with further 5 seconds timeout. The trigger force was set on 50g for starting the calculation. The cheese texture properties such us hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience were calculated using a macro using the Exponent 199 program.

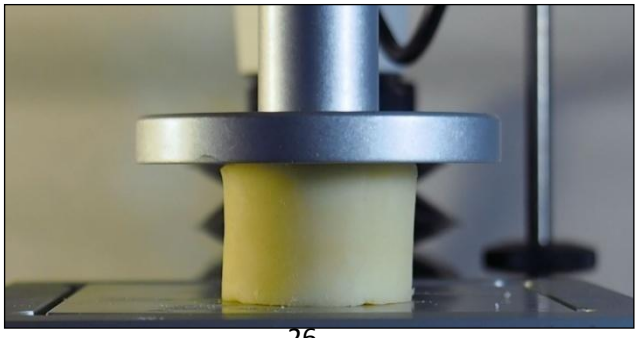


Figure 19: Texture analysis

2.5.3. Water activity Aw

The activity of water "aw" was determined at 20 ° C for 5 min on the surface of each sample while using AQUALAB 4TE as an activity measuring instrument which is based on the method of dew point with an absolute error of 0.003. The mean value of the water activity of each wafer was calculated from the estimated values at its surface and at the adjacent wafer surface.

Figure 20: Water activity determination

2.6. Bioactivity

2.6.1. Cytotoxicity

The cytotoxicity was carried out using the sulforhodamine B (SRB) assay according to the procedure previously described by Abreu et al., 2011 (62). This test was performed in order to evaluate the effects of the bioactive samples in non-tumor cells and also to establish a subtoxic concentration of these samples in the tumor cell line of Human adenocarcinoma cell line (CaCo2) obtained from European Collection of Cell Cultures (ECACC) CaCo2 cells. The normal cell line was a porcine liver primary culture (PLP2) which is established at the Mountain Research Centre.

Briefly, both cell lines were regularly preserved as adherent cell culture in RPMI-1640 comprising heat inactivated FBS (10%), glutamine (2mM), penicillin (100U/mL) and streptomycin (100µg/mL) and were further incubated at 37°C with humidified air and 5% with CO2.

To evaluate the cytotoxity, the cell lines were put in 96-well microplates, as well as the different samples dilutions to be analyzed (6.25-500µg/ mL), these were incubated at 37°C with 5% CO_2 for 48h. Just after incubation, the adherent cells were fixed by adding 10% of previously refrigerated trichloroacetic acid (100µl) and incubated at 4°C for 60 minutes. Subsequently, the microplates were washed with deionized water and dried.

The adhered SRB was eventually solubilized with 10mM Tris (200µl), and the absorbance was read in the microplate reader (ELX800) at a wavelength of 540 nm. The findings were then expressed as GI_{50} values (sample concentration inhibiting cell growth by 50%)

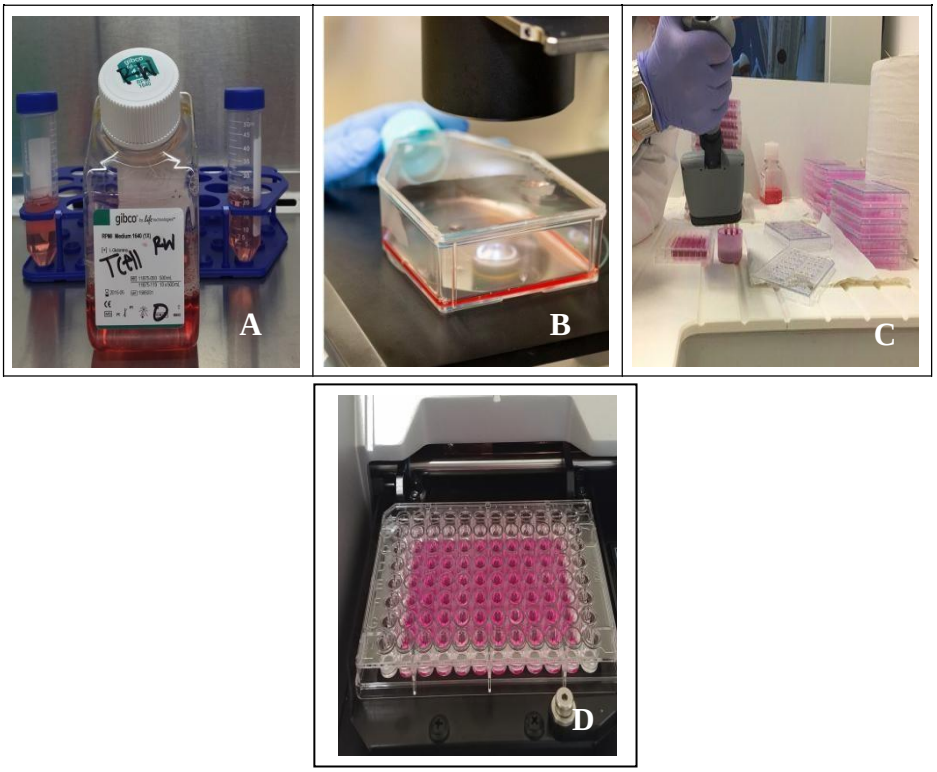


Figure 21 : Cytotoxicity: A- Cell suspension; B- Cell counting; C- adding sample in cells; D- absorbance at 540 nm.

2.6.2. *In vitro* cell transport assay in $CaCo_2$ cell cultures

Without FBS, glutamine (2mM), penicillin (100U/ml) and streptomycin (100µg / ml), the CaCo2 cell line was maintained in RPMI-1640 and incubated with humidified air and 5% CO_2 at 37°C. The cell lines were then placed on 44cm^2 insert membrane at a density of 5*10^5 cells per insert, for a pore diameter of 0.4µm.

The culture medium was replaced every 3 days, and before further experimental procedures, cells were allowed to differentiate for 21 days. Measuring transepithelial electrical resistance (TEER) (EVOM2); World Precision Instrument , Sarasota, FL) was measured by evaluating the integrity of the cell layer. Only inserts above 400 Ω have been used. The samples were applied to monolayers of $CaCo_2$ cells at the upper compartment concentration of incomplete medium (medium without added cholesterol) in 975µl. The microplate was incubated then with 5% CO_2 for 1h at 37°C.

For the quantification of cholesterol and ergosterol, the upper solution s well as the solution underneath the cell monolayer were therefore collected to determine the cholesterol/ ergosterol content absorbed by the CaCo2 cells (figure 23).

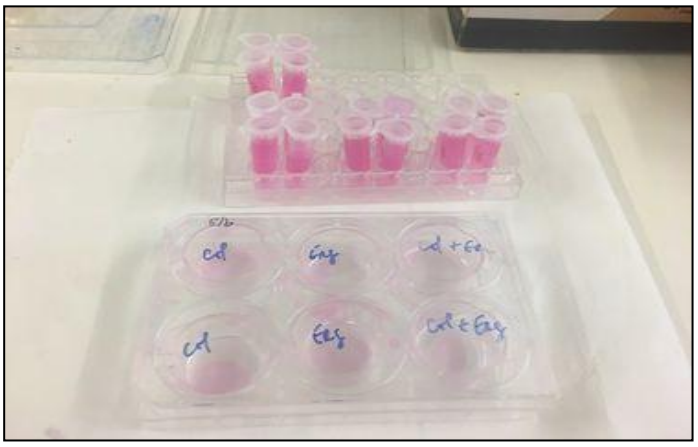


Figure 22:Addition of samples in Caco2 cells for cholesterol absorption

2.7. Microbial load

2.7.1 . General sample preparation

The cottage cheese sample preparation was carried out in accordance with the procedures of the International Organization for Standardization (ISO) 6887-1:2003, as defined by Carocho et al., 2019.(61)

For the preparation of the suspension, 10 g of cottage cheese was combined with 90mL of peptone water (PW) in stomacher bags and further homogenized in stomach equipment (ECN 710-0873, Italy) for 1 min at 300 units.

The suspensions obtained were then diluted in order to obtain dilutions of 10^{-1} to 10^{-12}, each dilution was analyzed in duplicate.

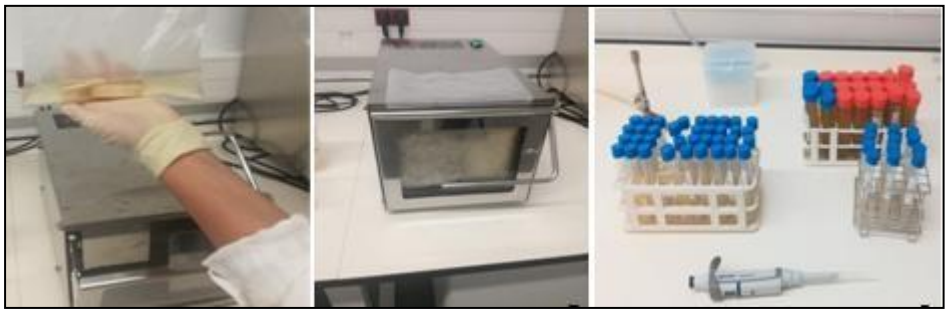


Figure 23: Sample preparation

2.7.2. Microorganisms analysis

Various microorganisms were analyzed in cottage cheese samples based on data relating to microorganisms, generally the most important ones that normally occur in cheese samples. Thus, aerobic mesophiles, enterobacteria, yeasts, molds and psychrotrophic bacteria were analyzed using a selective culture medium.

Aerobic Plate Count (APC): 1 ml of each prepared suspension was mixed with 20 ml of plate agar (PCA) according to the pour plate method, in duplicate (LOQ = 1 log CFU / g). Thereafter and in accordance with ISO 4833-2: 2013, the plates were incubated in an inverted position at 30 ºC for 72 h and counted.

Enterobacteria: 1 ml of each suspension was mixed with 20 ml of purple red bile lactose agar (VRBG), according to the plate method, in duplicate (LOQ = 1 log CFU / g). Thereafter and in accordance with ISO 4832: 2006, the plates were incubated in an inverted position at 30 ºC for 48 h and counted.

Psychrotrophic bacteria: 1 mL of each suspension was mixed with 20 mL of Man, Rogosa and Sharpe (MRS) agar, according to the plate method, in duplicate. after solidification of the medium, an additional 5 ml of medium was added to create anaerobiosis (LOQ = 1 log CFU / g). The plates were then incubated in an upright position at 22 ºC for 5 days and counted according to ISO 4832: 2006 (ISO 2006).

Yeasts and molds: In Petri dishes containing 20 mL of Dicloran Rosa Bengala Cloranfenicol Base (DRBC) agar, in duplicate (LOQ = 1.7 log CFU / g), 0.2 mL of each suspension was spread. In accordance with ISO 21527-2: 2008, the plates were then incubated in a vertical position at 25 ºC for 72 h for counting yeasts and 120 h for counting molds.

After incubation, the colonies count was expressed in CFU / g and carried out according to the following formula:

$$\frac{}{}$$

In which:

N: number of colonies per g or mL of sample;

Σc: sum of the colonies in the counted plates;

V: Volume of the suspension used;

n1: number of plates counted in the first countable dilution;

n2: number of plates counted in the second countable dilution;

d: first countable dilution;

2.8. Statistical analysis

All through the whole document, all data was expressed as mean ± standard deviation. The cottage cheese samples were analyzed by a two-way ANOVA with type III sums of squares using the SPSS Software, version 25, with the purpose of understanding the consequence of the addition of the pure ergosterol and mushroom extract to the cheeses, on the nutritional profile, fatty acids, lactic acid, external color and texture.

This multivariate general linear model deals with the two factors, maturation time (MT) and incorporation type (IT) as two independent factors, therefore its able to allow the effect of each factor to be analyzed independently and succeed to provide a closer look at their individual contribution towards the sample.

In case a significant interaction (<0.05) among the two factors (ST×IT) was recorded, they were simultaneously evaluated using the estimated marginal means (EMM) to obtain some general conclusions and tendencies.

When no significant interaction (>0.05) among the two factors (ST×IT) was recorded, each factor was evaluated independently using a simple Student's T test (for MT) or a Tukey's multiple comparison test (IT) when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. A Levene's test was used to evaluate the Homoscedasticity of the samples. The significance level used for all analyses was set at 0.05.

3. Results and discussion

3.1. Centesimal Composition and Soluble Sugar´s Profile

As stated in the previous section, for some analyzed parameters, a 2-way ANOVA was used, allowing for an individualized assessment of each factor. **Table 4** represents the nutritional profile of each cheese in g/100 g of fresh weight. The upper section displays the three analyzed times (0, 6 and 8 days), while the lower section shows the three cheese types, namely the control cheese (Control), the cheese incorporated with ergosterol (Ergosterol) and finally the cheese incorporated with *A. bisporus* extract (Extract). The values are represented as means of each storage time (ST) including all three incorporation types (I) in the upper section, and for the lower one, the means of I include all maturation times.

This type of representation allows the aforementioned independent analysis of each factor individually and thus, the standard deviations should not be regarded as an accuracy of an individual analysis as they include the variation of the non-fixed factor (ST or IT). If a significant interaction among these two factors is detected (ST×IT $p<0.05$), no multiple comparisons can be carried out, meaning that both factors, ST and IT, contributed, for each analysis, to the registered changes, hindering concrete conclusions, although general tendencies can be concluded from the Estimated Marginal Means (EMM) plots. Inversely, if this value is higher than 0.05, each factor was classified individually using either Tukey's or Tamhane T2 tests depending on the homoscedasticity of the distribution.

Table 4: Nutritional value and soluble sugars identified and quantified in the cottage cheese samples.

		Moisture (g/100 g fw)	Crude Fat (g/100 g fw)	Proteins (g/100 g fw)	Ash (g/100 g fw)	Carbohydrates (g/100 g fw)	Energy (Kcal)	Energy (Kj)	Lactose (g/100 g fw)	Glucose (g/100 g fw)	Total Soluble Sugars (g/100 g fw)
Storage Time (ST)	0 Days	70±4	14±2	8±1	2.5±0.8	8±5	186±16	780±67	18±2	0.4±0.6	18±2
	6 Days	70±5	13±2	8±1	2.3±0.8	8±4	182±15	761±64	18±4	0.3±0.5	18±4
	8 Days	74±3	11±2	7±1	2.2±0.6	8±3	161±19	674±81	16±1	0.4±0.7	17±2
p-value (n=3)	Tukey's HSD test	0.001	<0.001	<0.001	0.598	0.744	<0.001	<0.001	0.038	0.217	0.034
Incorporation (IT)	Control	69±6	11.7±0.7	8±2	2.4±0.6	11±4	183±26	764±110	20±3	-	20±3
	Ergosterol	73±2	13±4	7.0±0.4	2.3±0.8	7±3	172±19	719±82	15.7±0.9	-	15.4±0.8
	Extract	72±2	13±1	8.8±0.9	2.2±0.8	6±3	175±11	732±48	16±1	1.2±0.3	17±1
p-value (n=9)	Tukey's HSD test	<0.001	0.003	<0.001	0.798	<0.001	0.030	0.030	<0.001	<0.001	<0.001
ST×RD (n=27)	*p*-value	<0.001	<0.001	<0.001	0.023	<0.001	<0.001	<0.001	<0.001	0.201	<0.001

The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

In **Table 4**, it can be seen the nutritional profile of the cottage cheese incorporated either with ergosterol or *A. bisporus*, as well as the detected soluble sugars along a period of eight days, with an analysis on the day of manufacture, after 6 and 8 days. Analysing the cottage cheeses, as expected, moisture was the most abundant nutrient with over 69 g/100 g of fresh weight followed by fat, with carbohydrates and proteins showing very similar quantities. As expected, the incorporation of either the pure ergosterol or the mushroom extract did not have any type of influence in the centesimal composition of the cottage cheese. Observing Table 4 the *p*-value of the interaction among ST and IT was lower than 0.05, thus showing that both factors contributed to the variation, but none of them produced any significative changes to the nutritional profile. This was expected and ideal, due to not showing signs of change towards the nutritional profile of the highly appreciated cottage cheese that could constitute a change in the organoleptic profile.

Soluble sugars where detected by HPLC coupled to a refraction index detector taking advantage of the refraction of the light for detection. In this case, two sugars were detected, namely lactose and glucose. Lactose did not show any variation over the course of the 8 days, or was it influenced by the addition of ergosterol or the extract. Glucose was only detected in the cheese incorporated with *A. bisporus* extract, showing that the cheese did not have this sugar. Still, it is not expected that glucose would sweeten the cheese if added with the extract due to its very low concentration, under 1 g/100 g of fresh weight of cottage cheese.

The nutritional value of other cottage cheese' samples has already been described in previous studies (C. Caleja et al.2016), (63) in which the control cottage cheese presents, nutritionally, fat followed by proteins, as the most abundant constituents. The nutritional profile, in terms of fatty acids and soluble sugars, differed from the ones of this study, which can be justified by the different milk type and production of the cheese. For instance, their sample had less carbohydrates and a lower energetic value. These different results were expected since the cottage cheese was made in a different enterprise and in an industrial scale and in the cottage cheese samples described in the present study.

3.2. Fatty acids

Table 5 shows the contents in fatty acids present in the different cheeses over the 8 days of storage through gas chromatography coupled to a flame ionization detector.

Twelve individual fatty acids were detected, mainly saturated fatty acids, with only one monounsaturated and one polyunsaturated fatty acid, oleic (C18:1) and linoleic acids (C18:2), respectively. Thus, as verified in previous studies (Caleja C. et al., 2015) (64) the predominant fatty acids were, as expected, saturated ones followed by monounsaturated. Of all individual fatty acids, the most predominant were palmitic acid (±22%) with a considerable difference to the next most abundant one, oleic acid (±16%) and followed by capric (C10:0) and myristic acids (C14:0), practically in *ex aequo*. Considering the changes undergone by the fatty acids, in most cases there was significant interaction among the two factors (p-value < 0.05), and thus it was impossible to draw definitive conclusions. Still, in other cases a non-significative interaction was detected, but the changes were not statistically relevant in either of the factors, namely caproic acid (C6:0), (C8:0) caprylic acid, pentadecylic acid (C15:0) and oleic one (C18:1). In these cases, both factors showed influence, but this influence was not statistically significative .

Finally, in other cases, one factor had a significant influence on the outcome of each fatty acid, which allowed for a statistical classification. For instance, C14:0 is an example of this, where a significative increase is registered from day 6 to day 8, showing that time had a more significant effect on the registered variations than the incorporation of the ergosterol and the mushroom extract. Considering the total amount of saturated fatty acids (SFA), both the passage of time and the incorporation of ergosterol and the extract showed statistically significant changes. For instance, over time the amount of SFA increased, which is quite expected due to degradation of unsaturated fatty acids to saturated ones. Still, it seems like there was a significant difference among the cheese types in what concerns saturated fatty acids. Ergosterol and extract incorporated cheese showed a lower mount of SFA when compared to the control cheese, hinting at a preservation of the unsaturated fatty acids. Pure ergosterol incorporated cheese showed the lowest amount of SFA, with significant difference to the cheese with *A. bisporus* extract.

Table 5: Fatty acids profile of the cottage cheese samples incorporated with *A. bisporus* extract and ergosterol, represented in relative percentage over the 8 days of storage time.

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C17:0	C18:0	C18:1	C18:2	SFA
	0 Days	5±1	5.0±0.9	4.8±0.4	12±1	5.8±0.7	11.0±0.4a	1.1±0.1	22.0±0.5a	0.85±0.06	12±1	16.2±0.9	3.3±0.4	80±1a
Storage Time (ST)	6 Days	5.7±0.8	5.0±0.7	4.5±0.5	11.2±0.6	5.3±0.4	10.5±0.3a	1.15±0.08	22.0±0.5a	0.9±0.1	12.5±0.8	16.4±0.9	4±2	79±2a
	8 Days	4±1	4.3±0.7	4.4±0.3	12.1±0.4	6.1±0.1	11.9±0.4b	1.2±0.1	24±1b	0.9±0.2	12.1±0.6	15.5±0.5	2.5±0.8	81.9±0.7b
p-value (n=3)	Tukey's HSD test	0.039	0.073	0.058	<0.001	<0.001	0.009	0.053	0.005	0.005	0.010	0.015	<0.001	<0.001
	Control	5±1	5±1	4.6±0.6	12.2±0.9	5.9±0.6	11±1	1.1±0.1	24±2	0.9±0.2	12±1	16.0±0.6	2.3±0.8	82.0±0.4c
Incorporation (I)	Ergosterol	5.0±0.9	4.9±0.8	4.6±0.3	11.6±0.6	5.5±0.6	11±1	1.1±0.1	23±1	0.86±0.04	12±1	16.2±0.9	4±2	79.0±0.2a
	Extract	5±2	4.7±0.6	4.6±0.4	12±1	5.8±0.6	11±1	1.14±0.09	23.2±0.9	0.9±0.1	12.3±0.7	16±1	3.5±0.5	81.3±0.3b
p-value (n=9)	Tukey's HSD test	0.862	0.756	0.992	0.005	0.132	0.128	0.640	0.109	0.024	0.547	0.352	<0.001	<0.001
ST×RD (n=27)	*p*-value	0.017	0.060	0.079	<0.001	0.006	0.185	0.102	0.299	<0.001	<0.001	0.017	<0.001	0.140

SFA- Saturated fatty acids. In each row, different letters mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

3.3. Organic acids

Table 6 shows the profile in organic acids present in the different cheeses along the 8 days, detected by UFLC coupled to a diode array detector. Oxalic, malic, lactic, citric, fumaric and succinic acid were detected, being lactic and citric acids, the ones detected in higher amounts. Succinic acid was only detected in the cheese incorporated with the extract, which is in accordance with previous study done on the *A. bisporus* organic acids profile where the succinic acid was the most abundant component with 11478.4 mg/100 g fw (Gąsecka.M.et al ,2017) (65) , proving that *A. bisporus* was responsible for adding this acid to the cheese. In all but oxalic acid a statistically significative interaction among ST and I were detected not allowing the assessment of a clear influence of one factor over the other, proving the both the passage of time and incorporation type had influence on the changes undergone by the organic acids. Still, in the case of oxalic acid, by showing a p-value of 0.298, reveals that for this organic acid, both the storage time and incorporation had significative influences on the overall outcome.

In this case, a statistically significative influence was found from the 0 to the 6^{th} day while not showing this difference from the 6^{th} to the 8^{th}. This revels that the decrease of this acid could be due to *Lactobacillus* present in the cheese. Considering the incorporation types, it was also clear that the extract of *A. bisporus* also contributed considerable amount to this acid, as seen on the bottom part of Table 6, where the extract incorporated cheese had a higher statistically significative value when compared to the control and ergosterol incorporated cheese.

Table 6: Organic acids profile of the cottage cheese samples incorporated with *A. bisporus* extract and ergosterol, over the 8 days of storage time.

		Oxalic Acid (g/100 g fw)	Malic Acid (g/100 g fw)	Lactic Acid (g/100 g fw)	Citric Acid (g/100 g fw)	Fumaric Acid (g/100 g fw)	Succinic Acid (g/100 g fw)	Total Organic Acids (g/100 g fw)
Storage Time (ST)	0 Days	0.2±0.05a	0.41±0.08	0.6±0.1	0.6±0.1	0.03±0.01	0.1±0.2	2.0±0.5
	6 Days	0.05±0.08b	0.32±0.05	0.87±0.09	0.51±0.04	0.040±0.007	0.14±0.04	1.8±0.1
	8 Days	0.04±0.06b	0.14±0.04	0.6±0.1	0.33±0.06	0.034±0.004	0.06±0.09	1.2±0.1
p-value (n=3)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Incorporation (I)	Control	0.06±0.03a	0.3±0.2	0.8±0.1	0.5±0.2	0.043±0.009	-	1.7±0.5
	Ergosterol	0.04±0.05a	0.24±0.08	0.7±0.1	0.4±0.1	0.037±0.001	-	1.4±0.3
	Extract	0.2±0.1b	0.3±0.1	0.6±0.1	0.5±0.1	0.029±0.008	0.2±0.1	1.8±0.4
p-value (n=9)	Tukey's HSD test	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
ST×RD (n=27)	*p*-value	0.298	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

In each row, different letters mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

3.4. Texture and colour analysis

Table 7 shows the analyzed physical parameters of the cottage cheese incorporated with the hypocholesterolemic agents over the 8 days of storage. L*, a* and b* represent the external color of the cheeses using the CIELab color space where L* represents the lightness, a* the red-green, and b* the blue yellow tones. Of these, only a* showed a significative interaction among the two factors showing that both the passage of time and the incorporation of the hypocholesterolemic agents induced the changes. Still, as can be seen in Figure 24 the estimated marginal mean shows that a* decreased at day 8, revealing a tendency for all cheeses towards red. L*, lightness decreased over time with significant statistical difference from the 6^{th} to the 8^{th} day, showing that over time, the cheese became darker (Figure. 25). Furthermore, this darkening was less pronounced in the control cheese with a significant difference to the cheese with ergosterol, being the darkest cheese the one incorporated with the *A. bisporus* extract. Although there was a significant difference between the cheeses, the total variation was from 90 to 92, showing that it was not a very intense shift in color. Still, this darkening was expected due to the yellow color of the powdered mushroom extract.

Table 7: Texture and color parameters of the cottage cheese samples incorporated with *A. bisporus* extract and pure ergosterol over 8 days of storage time.

		L*	a*	b*	Hardness (g)	Adhesiveness (g.sec)	Springiness (%)	Cohesiveness (%)	Chewiness	Resilience (%)
	0 Days	91±1b	-1.4±0.4	11±2a	111±48	-18±4a, b	0.9±0.1	0.6±0.1b	47±8	0.21±0.06
Storage Time (ST)	6 Days	92±1b	-1.3±0.4	11±2a	168±94	-32±24a	0.9±0.5	0.3±0.1a	44±5	0.5±0.5
	8 Days	90.3±0.1a	-2.5±0.6	15±3b	94±40	-4±2b	0.9±0.1	0.44±0.08a	39±3	0.17±0.03
p-value (n=3)	Tukey's HSD test	0.001	<0.001	<0.001	<0.001	<0.011	0.837	0.003	<0.001	0.099
	Control	92.0±0.2c	-1.8±0.4	10±2a	196±82	-25±28	1.0±0.4	0.5±0.2	43±6	0.25±0.08
Incorporation (I)	Ergosterol	91.1±0.1b	-1.9±0.7	12±2a	86±17	-15±9	0.8±0.3	0.4±0.2	46±9	0.3±0.2
	Extract	90.4±0.2a	-1±1	16±2b	92±21	-14±10	0.9±0.2	0.4±0.2	40±4	0.4±0.6
p-value (n=9)	Tukey's HSD test	<0.001	0.193	<0.001	<0.001	0.112	0.172	0.769	0.001	0.731
ST×RD (n=27)	*p*-value	0.093	0.030	0.345	0.001	0.069	0.002	0.697	<0.001	0.624

In each row, different letters mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

Table 7 also shows the different dimensions of texture after a texture profile analysis (TPA) composed of two compressions of the cheeses. Apart from adhesiveness and resilience, in all other dimensions there was a significant interaction between ST and I, and thus no definitive statistical differences could be sought. The first dimension, adhesiveness, is defined as force required to remove the cheese sample that adheres to the mouth surface. (Paula and Conti-Silva, 2014) (66) Regarding this dimension, ST showed a significant effect, showing a significative decrease from the 6th to the 8th day. In terms of cohesiveness (degree to which the cheese sample deforms before rupturing), the inverse was detected, showing that cohesiveness increased over time. Overall, ST had a higher influence on the changes in texture when compared to the influence of ergosterol and mushroom extract.

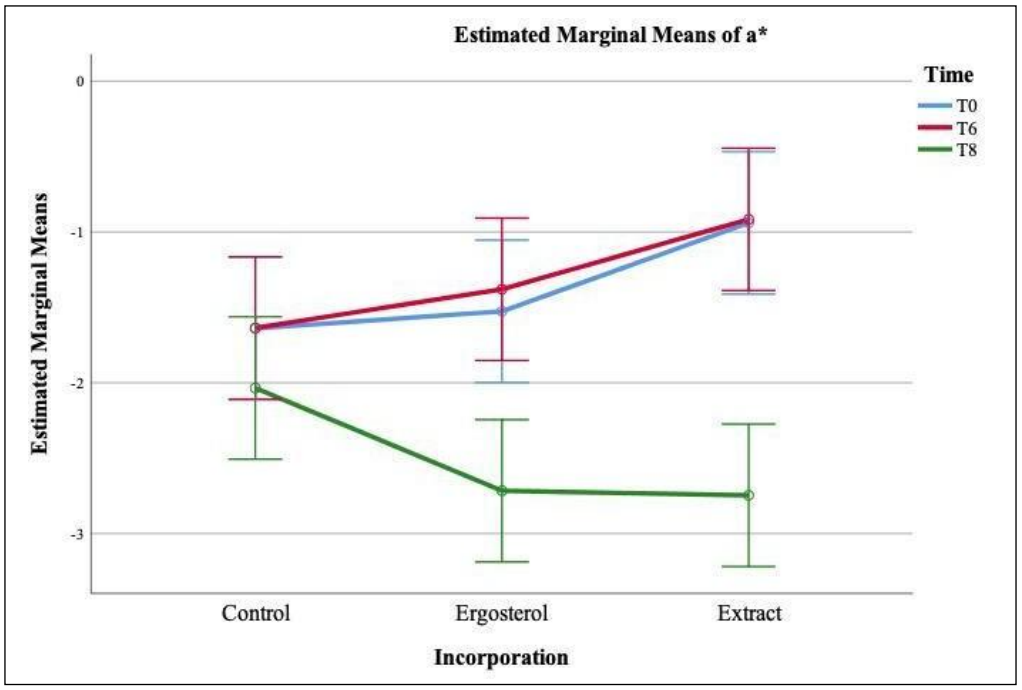


Figure 24: Estimated marginal mean of a* showing a slight decrease in this value in the cheeses after 8 days.

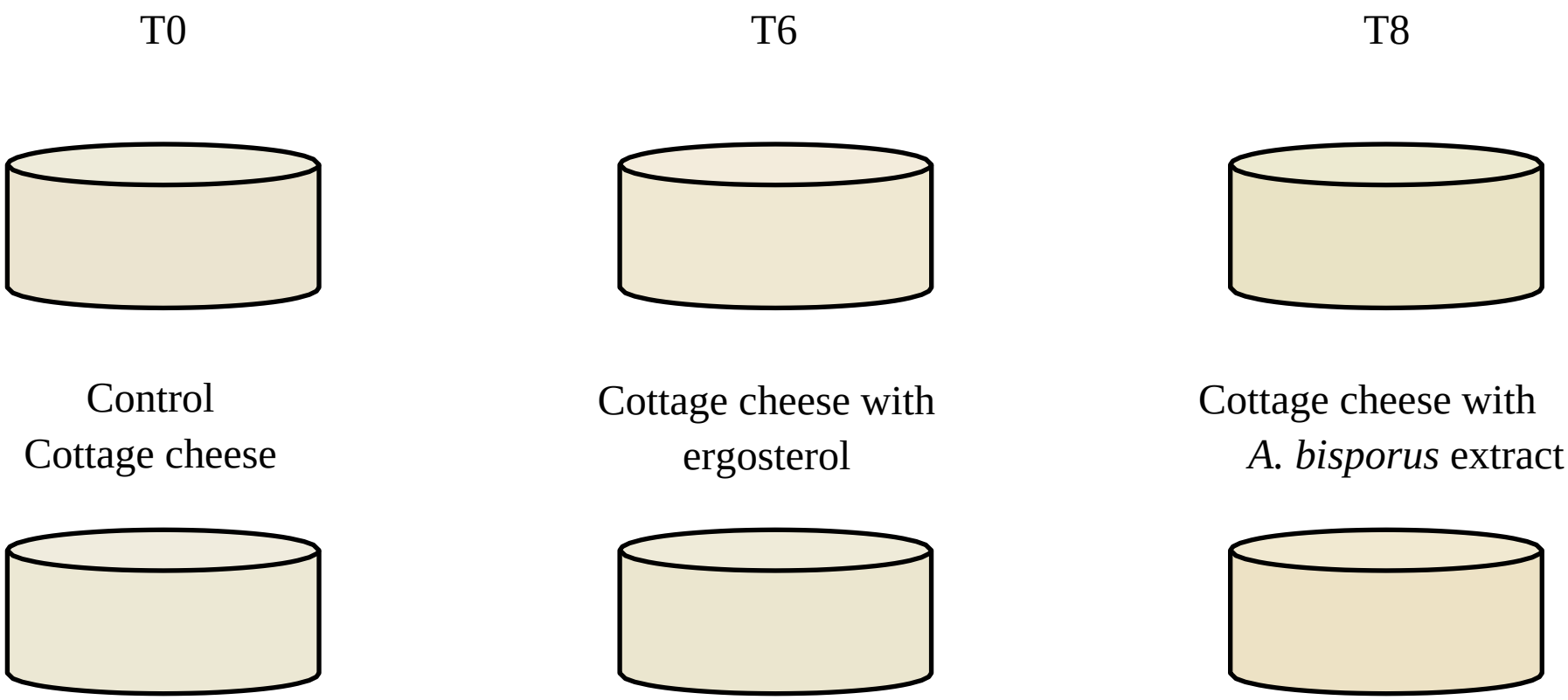


Figure 25: Representation of the external colors of all the cottage cheeses along the storage time (top row) and the difference among the cheese with each incorporation (bottom row)

3.5. Cholesterol and ergosterol contents in cottage cheeses

Before the bioactive analysis of the samples, the cholesterol and ergosterol contents present in the developed cheeses were identified and quantified, so that, it was possible to infer the amount of cholesterol present in the cottage cheese and monitor the amount of ergosterol that was effectively incorporated (**Table 8**).

Table 8: Cholesterol and ergosterol quantification in the Cottage cheeses.

	Control Cottage Cheese	CCE	CCEXT
Cholesterol (mg/100g fw)	29.45±0.05	27.22±0.02	28.53±0.04
Ergosterol (mg/100g fw)	-	175±4	94±3
Ergosterol losses (%)	-	42%	34%

fw: fresh weight

From the ergosterol and cholesterol quantifications expressed in Table 4, it can be seen that the cholesterol content present in the sheep cottage cheese rounds from 27.22±0.02 to 29.45±0.05 mg/100g Fw. Concerning the incorporation of *A. bisporus* extract and pure ergosterol, it can be stated that considering the initial content incorporated in the cottage cheese, the incorporation was not 100% efficient, since ergosterol losses were verified. From the incorporated content of pure ergosterol (300 mg/100g fw), only 175±4 mg/100g fw were quantified, presenting a loss of 42%, while from the incorporated content of *A. bisporus* extract (ergosterol = 144 mg/100g fw), a total of 94 mg/100g fw were quantified, meaning a loss of 34% of ergosterol. These ergosterol losses can be attributed to cottage cheese making process, precisely in the step, in which the cottage cheese is placed in shapes with tiny holes allowing the removal of the excess of whey. During this process, part of the *A. bisporus* extract, as also part of the ergosterol are dragged by the whey from the shapes, thus decreasing the incorporation efficiency. Ergosterol presents very low to none solubility in aqueous medium, a fact that can also difficult the ergosterol resistance to the whey removal. Nevertheless, it is possible to conclude that the losses in the incorporation with *A. bisporus* extract are lower, a fact that can be explained by the combination of ergosterol with other molecules that allows it to resist to the whey removal.

This ergosterol loss can be overcome, by using stabilizing technologies that can protect the ergosterol and increase its solubility in lipophilic medium, thus decreasing the observed losses. Besides that, the amount of extract can also be increased in order to offset the loss. Among the two analyzed incorporated ingredients, the *A. bisporus* extract appears as the most suitable one for industrial application, since it´s preparation presents significantly lower costs comparing with pure ergosterol, a purified molecule.

3.6. Analysis of cytotoxicity and cholesterol absorption

The cytotoxicity analysis of the *A. bisporus* extract, pure ergosterol and cholesterol was carried out in the normal cell line PLP2 and in the tumor CaCo2 cell line. This assay was performed with the aim of evaluating the safety of this agents for normal cells and also to select a subtoxic concentration to apply in the CaCo2 cells in the cell transport assay. According to the obtained results, for the PLP2 cell line, the *A. bisporus* extract presented no effect with a GI_{50} > 400 μg/mL, the pure ergosterol presented a GI_{50} of 91±13 μg/mL and cholesterol a GI_{50} of 109±6 μg/mL. These results are in agreement with previous works, namely with Francisco et al,. 2018 (67) that obtained similar results for the cytotoxic effects of *A. bisporus* extract and pure ergosterol.

Concerning the cytotoxicity of these agents to the Caco2 cell line, the *A. bisporus* presented a GI_{50} value of 212±9 μg/mL, followed by cholesterol (GI_{50} = 88±2 μg/mL) and ergosterol (GI_{50} = 59±3 μg/mL).

From the results exhibited regarding the cytotoxic effects of the *A. bisporus* extract and pure ergosterol, a subtoxic concentration of 50 μg/mL of ergosterol was selected and applied in the cell transport assay. The same concentration was selected as the subtoxic concentration of cholesterol, based on the fact that these molecules are direct competitors in the absorption process, given their similar chemical structure. Therefore, for the cell transport assay, different samples were prepared with culture medium as follows: i) control samples: ergosterol (50 μg/mL); cholesterol (50 μg/mL); sheep cottage cheese amount correspondent to 50 μg/mL of cholesterol; *A. bisporus* extract amount that allows a concentration of 50 μg/mL of ergosterol; ii) sheep cottage cheese with pure ergosterol (CCPE) allowing a concentration of 50 μg/mL of ergosterol; sheep cottage cheese with *A. bisporus* extract (CCEXT) allowing a concentration of 50 μg/mL of ergosterol. The results obtained in the cell transport assay can be observed in Figures 26 and 27.

When analyzing the control sample with cholesterol solution, it is possible to distinguish that 54.44% of the applied cholesterol was detected in the upper compartment,

while 43.89% was detected in the underneath one. Also, when analyzing the control samples with ergosterol, both ergosterol solution and the *A. bisporus* extract present a similar behavior, being possible to quantify about 43.75% for ergosterol solution and 46.27% for the *A. bisporus* extract in the upper compartment; and about 12.05% and 13.93% in the underneath compartment, respectively. It indicates that, like the action of cholesterol, ergosterol is not completely emitted by the CaCo2 cells. In addition, it is noted that ergosterol is less absorbed by Caco2 cells than cholesterol.

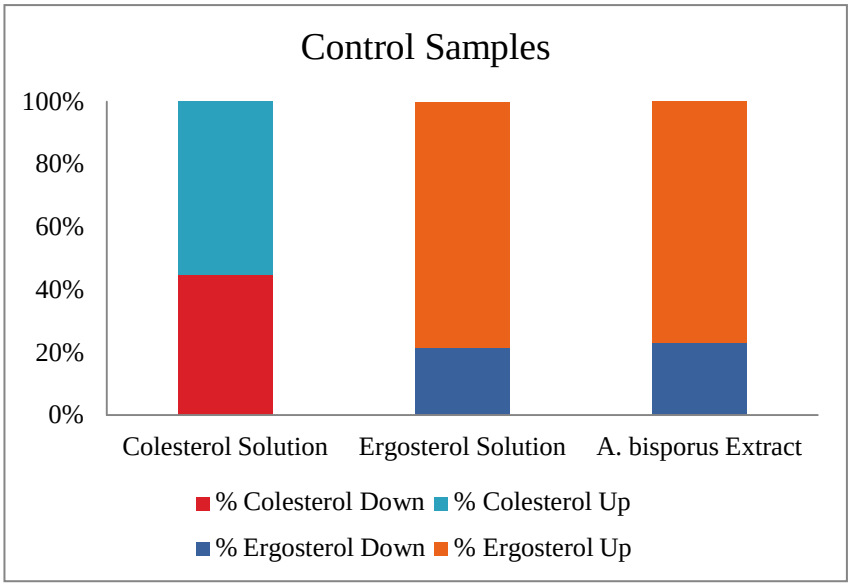


Figure 26: Cholesterol and ergosterol quantification in the upper and underneath compartments of the control samples applied to the CaCo2 cell line

Figure 27 reveals the cholesterol and ergosterol quantification in the upper and underneath compartments of the cottage cheese samples after application in the cell transport assay. For the control cottage cheese, is was possible to verify that from the total cholesterol content of 29.45 mg/100g fw, 66.55% was not absorbed, being quantified in upper compartment and 43.89% was absorbed by the CaCo2 cells, quantified in the underneath one. These results mean that the cholesterol present in the cottage cheese is not all released by the cells, since the sum of the upper and the underneath compartments does not correspond to the total amount of cholesterol present in the samples, thus being trapped inside the cells. As stated by other authors, these can be due to the accumulation of cholesterol and ergosterol in the intracellular phase or to the transformation of ergosterol in other ergosterol derivatives that were not analyzed (Gil-Ramírez et al., 2014) (68).

Regarding the cottage cheese samples incorporated with *A. bisporus* extract, 57.97% and 20.74% of the cholesterol content was quantified in the upper and underneath compartments, respectively. Concerning the cottage cheese with pure ergosterol, 60.78% and 26.78% of cholesterol was quantified in the upper and underneath compartments, respectively. These satisfactory results corroborate the ability of ergosterol in reducing the cholesterol absorption by direct competition due to the similar structure with cholesterol. Thus it was possible to obtain a significant reduction in cholesterol absorption in the cottage cheese samples incorporated with *A. bisporus* extract (reduction of 30.24%) and with pure ergosterol (reduction of 21.1%).

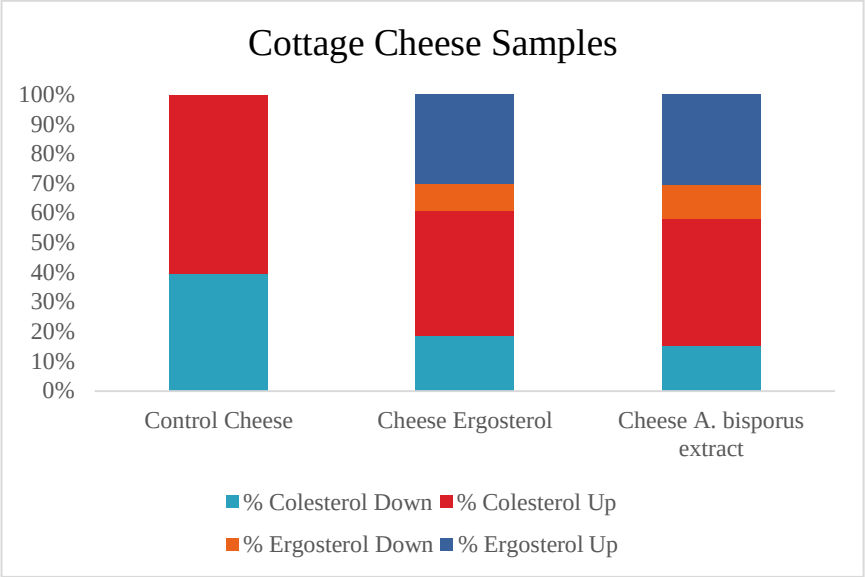


Figure 27: Cholesterol and ergosterol quantification in the upper and underneath compartments of the cheese samples applied to the Caco2 cell line

By analyzing the ergosterol behavior, it can also be stated that in the cottage cheese incorporated with pure ergosterol, 43.22% was quantified in the upper compartment, while 13.02% was quantified in the underneath one. A similar behavior was obtained for the *A. bisporus* extract, quantifying a total of 41.14% of ergosterol in the upper compartment and 15.55% in the underneath one, for the cheese with *A. bisporus* extract incorporation.

Therefore, the sample of cheese incorporated with *A. bisporus* extract showed a higher ability to decrease the absorption of cholesterol, a fact that may be due to the presence of other molecules in the extract besides ergosterol, which can bind to cholesterol and increase its molecular weight, while preventing passage through the cell membrane.

These results clearly corroborate the ergosterol capacity in the reduction of cholesterol absorption as well as its ability to compete with cholesterol. Nevertheless, this study was focused on the cholesterol absorption, and the results are in agreement with other studies that also enriched food with plant stanols that have gained a prominent position in decreasing cardiovascular risk by dietary means, and verified that in the presence of the extract, the cholesterol content released by the cells was lower in various populations and patient groups Katan et al., 2003. (69)

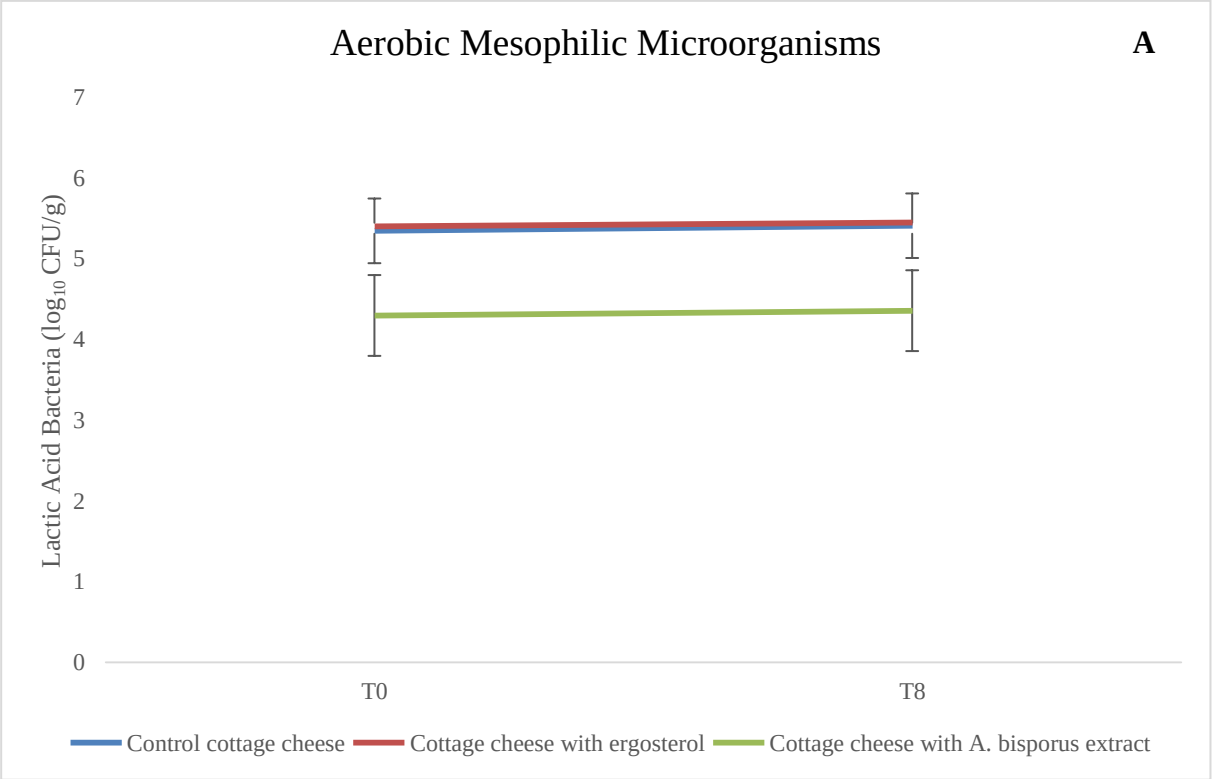
Also, Ergosterol was as effective as some phytosterols such as sitosterol, in the displacement of cholesterol when applied to the food matrix. As stated by other authors, fungal sterols also impaired proper cholesterol transport through Caco2 monolayers. (Gil-Ramírez et al., 2014).

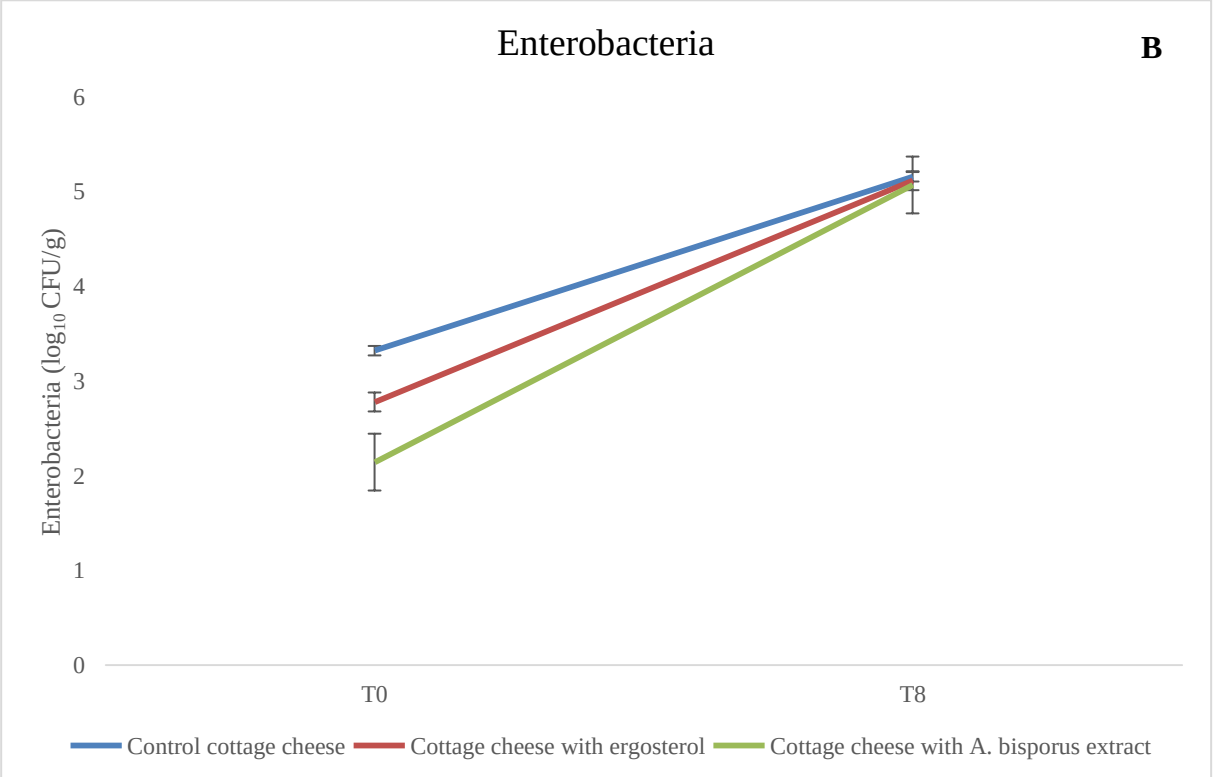
The Journal of functional foods (2019), also stated that micelles formation assay *in vitro* clearly demonstrated that ergosterol could significantly inhibit the entry of cholesterol into micelles. Therefore, the cholesterol-reducing effect of ergosterol was regulated by suppressing intestine cholesterol absorption and promoting the excretion of fecal cholesterol via modulating the expression of hepatic cholesterol-related genes.

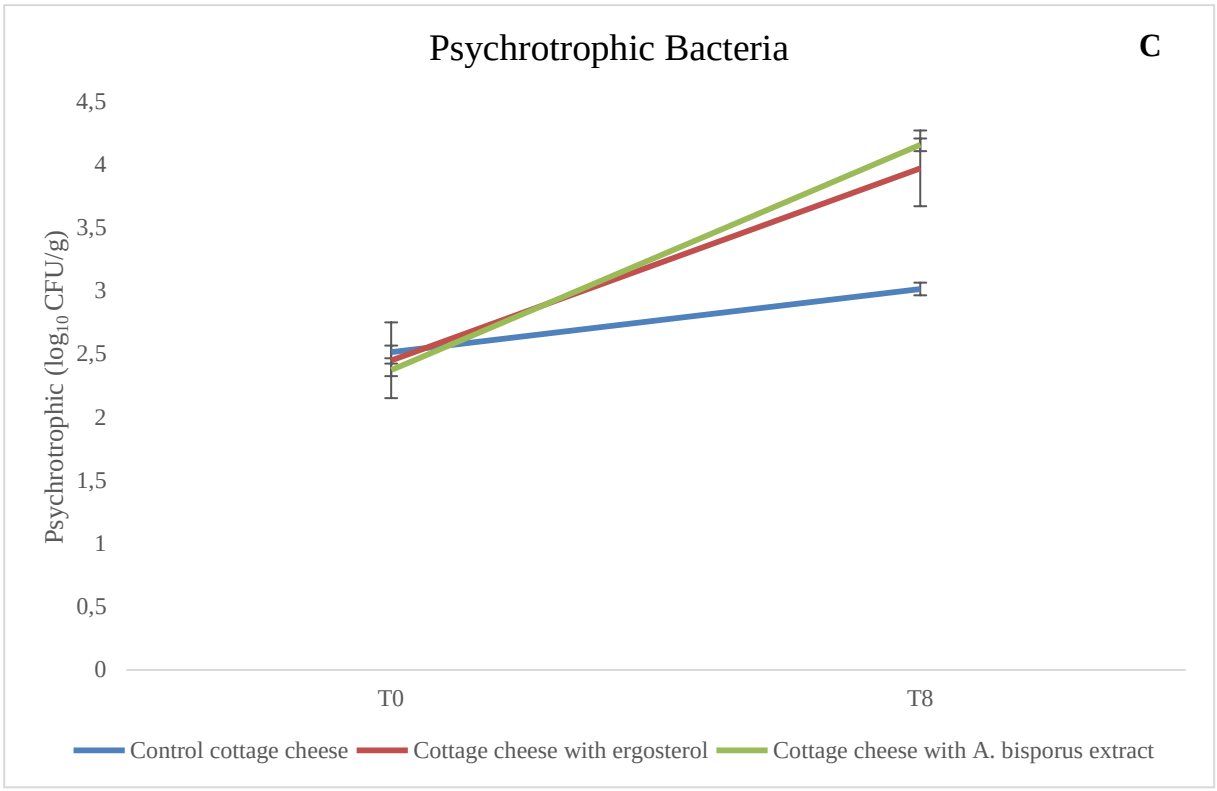
With an overview of the above described results and taking into consideration the industrial application and the costs to develop these hypocholesterolemic agents, it can be stated that the *A. bisporus* extracts appears as the most promising solution to be applied for this purpose, demonstrating the stronger hypocholesterolemic potential. It is an extract obtained from mushroom´s biowaste, allowing the valorization of this raw material to obtained high valuable bioactive molecules. When compared with pure ergosterol (a purified molecule), this extract doesn´t need a purification step, thus significantly reducing the costs to develop this hypocholesterolemic agent, besides using green solvents for its obtention.

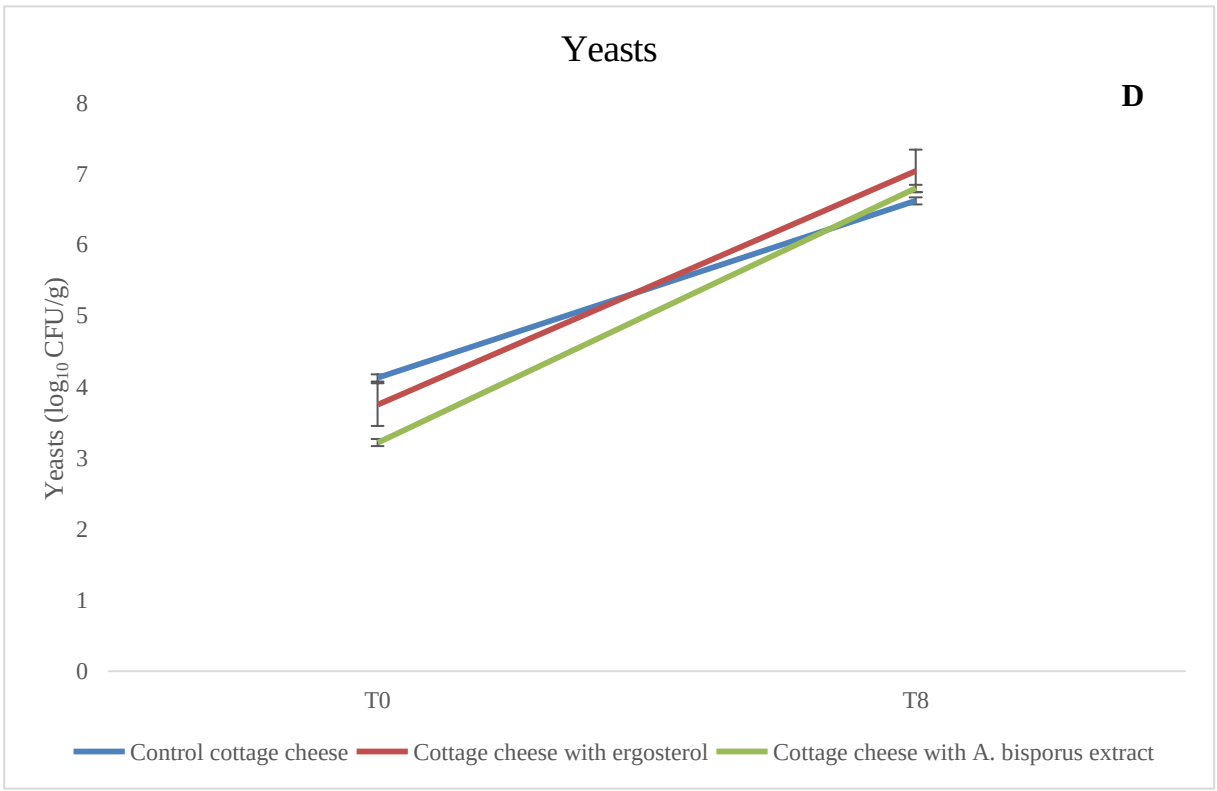
3.7. Microbiological analysis

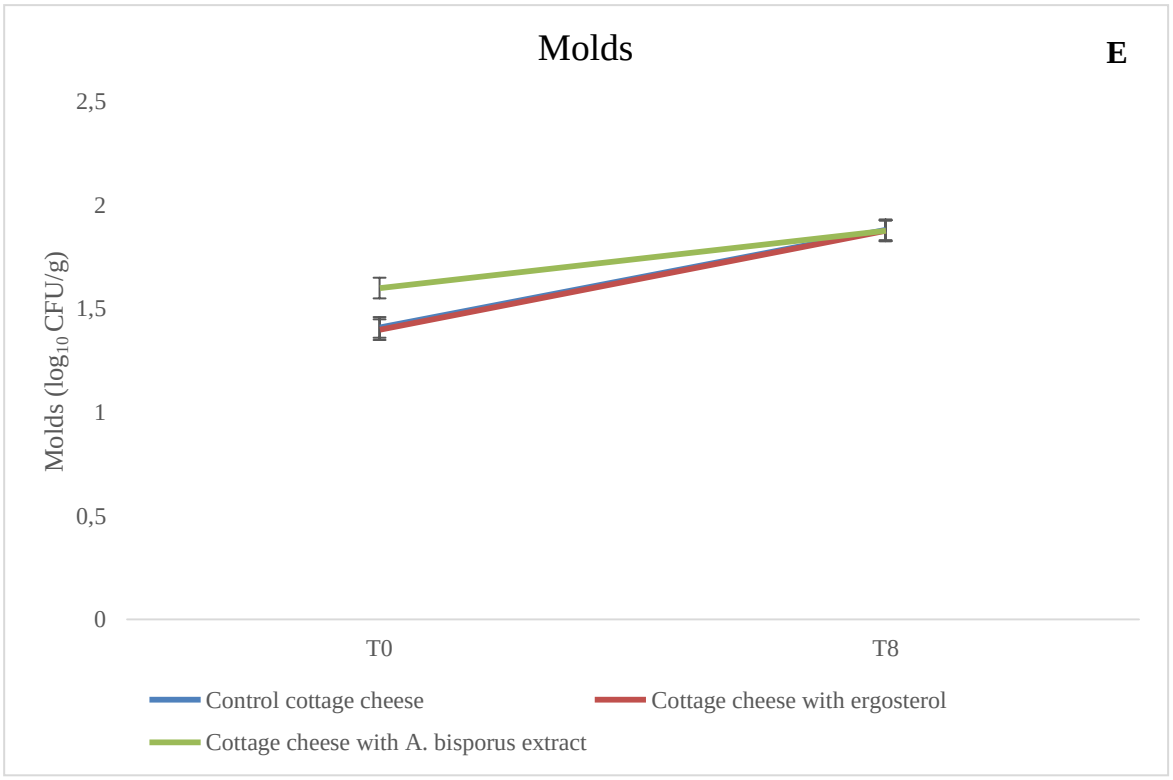
The microbial analysis was performed in the cottage cheese with *A. bisporus*, the cottage cheese with ergosterol and in a control cottage cheese without any incorporation. This assay was carried out in order to verify the effect of the incorporations in the microorganism growth in this type of foodstuff. For that, different microorganisms usually considered as responsible for the cheese deterioration and pathogenic were analyzed, namely: Total aerobic mesophiles, enterobacteria, Psychrotrophic bacteria, yeasts, molds and *Staphylococcus aureus* along a shelf life of 8 days.


Aerobic Mesophilic Microorganisms
A
Lactic Acid Bacteria (log10 CFU/g)
7
6
5
4
3
2
1
0
T0
T8
Control cottage cheese
Cottage cheese with ergosterol
Cottage cheese with A. bisporus extract


Enterobacteria
B
Enterobacteria (log10 CFU/g)
6
5
4
3
2
1
0
T0
T8
Control cottage cheese
Cottage cheese with ergosterol
Cottage cheese with A. bisporus extract


Psychrotrophic Bacteria
C
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Psychrotrophic (log10 CFU/g)
T0
T8
Control cottage cheese
Cottage cheese with ergosterol
Cottage cheese with A. bisporus extract


Yeasts
D
8
7
6
5
4
3
2
1
0
Yeasts (log10 CFU/g)
T0
T8
Control cottage cheese
Cottage cheese with ergosterol
Cottage cheese with A. bisporus extract



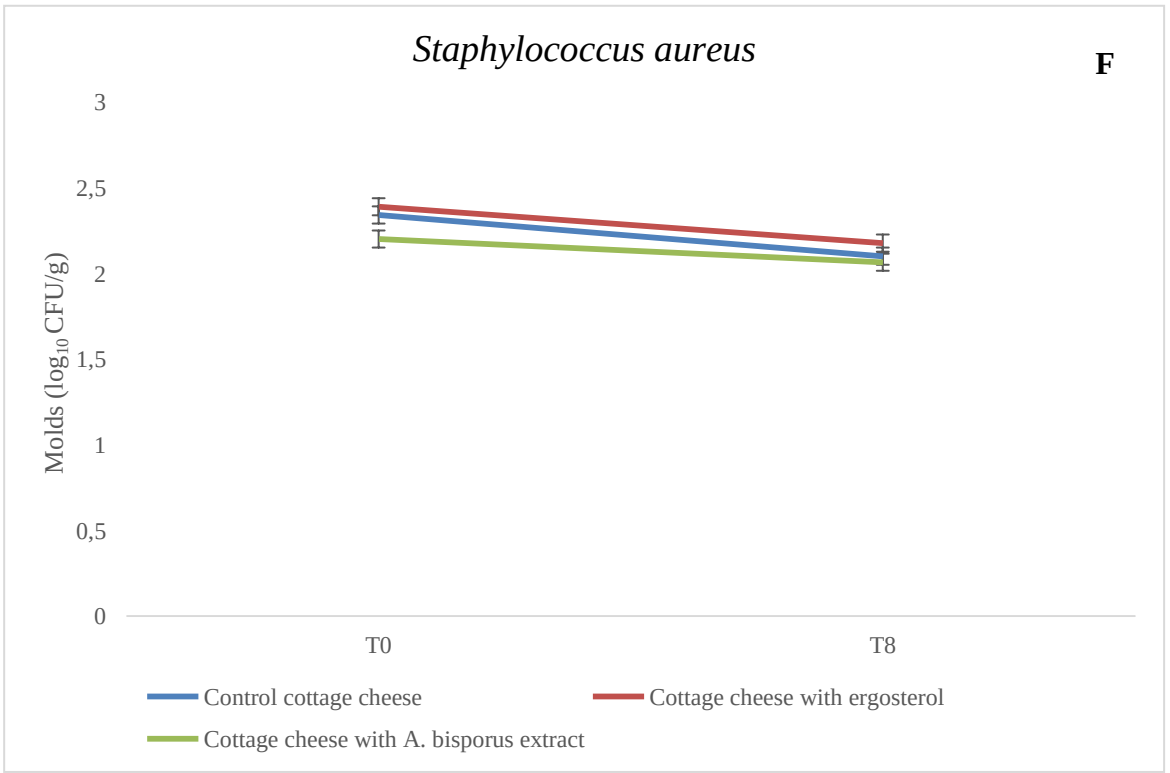


Figure 28: Microbial load of the cottage cheese samples over the shelf life period

By the obtained results, it can be seen that the incorporation of the *A. bisporus* extract and the pure ergosterol, in general it didn´t significantly influence the normal microbial growth of the cottage cheese samples (Figure 28) during the time. Analyzing the Time 0 and concerning the

aerobic mesophilic microorganisms, the enterobacteria, yeasts, molds and *S. aureus* it can be seen from Fig 28A, B, E, F, that in Time zero, a significative difference was found with the extract presenting lower contents in these microorganisms when compared with the control cottage cheese and the one incorporated with pure ergosterol, except for the molds (Fig. 28 D) where the cottage cheese with the extract incorporation presented a significative difference comparing with the other formulations exhibiting a higher content in this microorganisms; and for the psychrotrophic bacteria that there was no significative differences between the different formulations (Fig. 28 C).

On the other hand, when comparing the microorganism's growth over the time, the significative differences and the contents in these microorganisms were also maintained over the time, with the cottage cheese with the *A. bisporus* extract incorporation exhibiting the lower contents.

Regarding the other microorganisms it was possible to verify that the contents of each microorganism increased over the time. Regarding the enterobacteria, a significant increase was verified after 9 days of storage, with all the formulations presenting a count higher than the one counted in Time 0. Nevertheless, this increase in the enterobacteria is not a result from the incorporation of the extract, neither from the incorporation of the pure ergosterol, since it is possible to observe that the cottage cheese with no incorporations also presented a significant increase in these microorganisms (Fig 28 B). Besides the contents increased, they still under the legal and allowed limits defined by the Commission Regulation (EC) No 2073/2005 which is 10^5UFC/g.

Among the psychrotrophic bacteria, an increase was also verified over the time in all the developed formulations. This increase in the content of lactic bacteria is benefic and results from the consumption of lactose as it was observed in Table 4, originating also an increase of lactic acid, as it can be observed In Table 6.

Concerning the yeasts, molds and s. aureus, also a similar behavior was verified between the cottage cheese (control) and the other developed cottage cheese formulations.

With the observed results it can be observed that the incorporation of *A. bisporus* extract, and the incorporation of pure ergosterol, did not cause significant changes in the normal growth of the microorganisms present in cottage cheese over the time, meaning that it has no interference with the shelf life of the cottage cheese and does not cause the cheese degradation by increasing the bacterial and fungal contents.

4. Conclusions and perspectives

Functional foods have been the target of an intense investigation by researchers, highly demanded by consumers as also with increasingly strict regulatory processes applied to agro-industries. Despite dairy products present a broad history of use, in the last years a high demand by consumers for enhanced-dairy foods with additional health-promoting abilities has been observed. These functional foods have increasingly become attractive alternatives to prevent and treat hypercholesterolemia and reduce the risk for cardiovascular disease.

The present dissertation was focused on the development of cottage cheese with hypocholesterolemic agents, namely pure ergosterol and *A. bisporus* extract enriched in ergosterol, in collaboration with "Queijaria Vaz" that was a partner in the development of the final cheese formulation. In view of these results, it is possible to conclude that storage exerted the main effect on the nutritional composition of cottage cheese. The incorporation of the two hypercholesteremic agents did not cause significant alterations in the cottage cheese.

Also, the incorporation of these two formulations did not interfere in the normal growth of microorganisms which was expected and desired due to the incorporation having the sole effect of being hypocholesterolemic. Despite having an increase of enterobacteria in the cheeses with the incorporation of the two agents, this increase was also verified for the control cottage cheese. Furthermore, the darkness in the cheese is less pronounced in control and cheese incorporated with ergosterol; the one incorporated with *A. bisporus* present the darkest color due to the yellow color of the extract.

Regarding the hypocholesterolemic activity is was verified a strong capacity of the *A. bisporus* extract and pure ergosterol in reducing the cholesterol absorption by the CaCo2 cells, highlighting the stronger capacity of the extract, a fact that may be due to synergisms of other molecules with ergosterol that avoid the absorption of cholesterol.

Moreover, the main goal of the present work was successfully achieved; it was possible to develop a functional dairy product with hypocholesterolemic capacity; in line with the consumer´s and the industry's demands.

As perspectives, this work proved to be of great interest and worthy of proceeding to further studies, such as *in vivo* studies in order to corroborate this laboratory and *in vitro* potential. Sensorial analysis also must be conducted in order to evaluate the consumer acceptance of the developed cheeses. The introduction of innovation and development of new

foodstuffs is always welcomed in the food industry, and moreover if these innovations bring bioactive properties to traditional foodstuffs.

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