Development of bio-waste apple flour with potential application in the bakery industry

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ABSTRACT

Food loss and waste has attracted the attention of many sectors worldwide due to negative environmental and economic effects. In addition, several studies referenced in the literature have been highlighting this type of bio-waste due to its nutritional content and concentration of bioactive compounds. These industry surpluses can be exploited and used as natural ingredients and can be incorporated by the food, pharmaceutical and/or industry, or cosmetics by valuing bio-waste without recovery, thus benefiting the environment and the economy and producing functionalized products with high added value.

In this sense, this study aimed at the nutritional, chemical and bioactive evaluation of flour obtained from apples discarded as bio-waste due to its low caliber or non-standard shape. After the characterization, the flour was incorporated into two different types of bread (wheat bread and corn bread) and the effect of this incorporation was evaluated over the shelf life to validate its incorporation as a healthier alternative in the bakery industry. In a more in-depth way, for chemical and nutritional characterization, the official AOAC analysis methodologies were followed. The nutritional evaluation of the flour highlighted an interesting protein and ash content. Three free sugars were identified in the analysed flour, with fructose being the major sugar (8.9 ± 0.5 g/mg) and five organic acids, of which malic acid was the most prevalent (20 ± 1 mg/g). Phenolic compounds were determined by high performance liquid chromatography coupled to a diode detector and mass spectrometry detector (HPLC-DAD-ESI/MS) and different bioactivities (antioxidant, antimicrobial and antiproliferative activities) were analysed. Apple flour had seven phenolic compounds in its composition with a prevalence of phenolic acids.

After the characterization of apple flour, it was incorporated into wheat bread and corn bread. The effects of this incorporation on the colour and texture of the breads, on the nutritional composition, sugars, and fatty acids as well as the microbial load were evaluated over the shelf life and compared to traditional breads. The addition of apple flour appears to slightly increase the colour intensity of the bread, which is attractive in the bakery industry. However, in an overview, it does not seem to cause very significant changes in the texture or nutritional composition, which meets the expectations of the bakery industry, which intends to maintain the traditional characteristics of a product that is consumed and appreciated worldwide. In terms

of microbial load, apple flour seems to show some protection against total aerobic mesophiles and molds at least up to 3 days of storage.

In a global evaluation, the nutritional and chemical composition of the apple flour obtained in this study, makes it a viable alternative for application in the bakery and pastry industry, thus creating alternative and health enhancing products for the consumer. Additionally, the reuse of apple bio-waste will allow the recovery of waste discarded annually without added value, in addition to contributing to a positive impact on the economy and the environment.

Keywords: Circular economy, Apple flour, Bio-waste, Bioactivities, Bread.

RESUMO

A perda e o desperdício de alimentos têm atraído a atenção de muitos setores a nível mundial devido aos efeitos ambientais e económicos negativos. Para além disso, diversos estudos referenciados na literatura têm vindo a destacar este tipo de bio-resíduos devido ao seu conteúdo nutricional e conteúdo de compostos bioativos que podem ser explorados industrialmente e, utilizados como ingredientes naturais podendo ser incorporados pela indústria alimentar, farmacêutica e/ou cosmética valorizando bio-resíduos sem valorização, beneficiando desta forma o ambiente e a economia e produzindo produtos funcionalizados de elevado valor agregado.

Neste sentido, este estudo pretendeu a avaliação nutricional, química e bioativa de farinha obtida a partir de maças descartadas como bio-resíduos devido ao seu baixo calibre ou forma não standard. Depois da realizada a caracterização, a farinha foi incorporada em dois tipos de pães distintos (pão de trigo e pão de milho) e o efeito dessa incorporação foi avaliado ao longo do tempo de prateleira de forma a validar a sua incorporação como alternativa mais saudável na indústria panificadora. De um modo mais aprofundado, para a caracterização química e nutricional foram seguidas as metodologias oficiais de análise AOAC. A avaliação nutricional da farinha destacou um interessante conteúdo de proteínas e cinzas. Foram identificados três açúcares livres na farinha analisada sendo a frutose o açúcar maioritário (8.9±0.5 g/mg) e cinco ácidos orgânicos dos quais se destacou o ácido málico com maior prevalência (20±1 mg/g). Os compostos fenólicos foram determinados por cromatografia líquida de alta eficiência acoplada a detetor de díodos e detetor de espetrometria de massa (HPLC-DAD-ESI/MS) e foram analisadas diferentes bioatividades (atividades antioxidante, antimicrobiana e antiproliferativa). A farinha de maçã apresentou sete compostos fenólicos na sua composição com prevalência de ácidos fenólicos.

Após a caracterização da farinha de maçã, esta foi incorporada em pães de trigo e pães de milho. Os efeitos dessa incorporação na cor e textura dos pães, na composição nutricional, açúcares e ácidos gordos bem como a carga microbiana foram avaliados ao longo do tempo de prateira e comparando com os pães tradicionais. A adição de farinha de maçã parece aumentar ligeiramente a intensidade da cor do pão, o que é atraente a nível da indústria de panificação. No entanto, numa visão geral parece não provocar alterações muito significativas na textura ou

composição nutricional o que vai de encontro às expetativas da indústria de panificação que pretende manter as características tradicionais de um produto mundialmente consumido e apreciado. A nível da carga microbiana, a farinha de maçã parece demostrar alguma proteção contra mesófilos aeróbicos totais e os bolores pelo menos até aos 3 dias de armazenamento.

Numa avaliação global a composição nutricional e química da farinha de maçã obtida neste estudo, torna-a uma alternativa viável para aplicação na indústria da panificação e pastelaria criando desta forma produtos alternativos e potenciadores de saúde para o consumidor. Adicionalmente o reaproveitamento de bio-resíduos de maçã permitirá a valorização de resíduos descartados anualmente sem valor acrescentado para além de contribuir para impacto positivo na economia e no ambiente.

Palavras-chave: Economia circular, Farinha de maçã, Bio-resíduos, Bioatividades, Pão.

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*a**	Red/green chromaticity
AGS	Gastric adenocarcinoma
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
*b**	Blue/yellow chromaticity
CFU	Colony Forming Unit
CH_0	Optical haemolysis density completes at 0 min
DAD	Diode Array Detector
DM	Dry Matter
DMEM	Culture medium for animal cells
DPPH	2,2-diphenyl-1-picrylhydrazyl
dw	dry weight
ESI/MS	Electrospray Ionization Mass elestroscopy
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FID	Flame ionization detector
fw	fresh weight
GAE	Gallic Acid Equivalent
GC-FID	Gas chromatography with flame ionization

GI_{50}	Concentration that inhibits 50% of cell growth
HBSS	Hank's saline solution
HPLC	High Performance Liquid Chromatography
Ht_{50}	50% haemolytic time (min)
IC_{50}	Extract concentration corresponding to 50% of the antioxidant activity
INT	*p*-iodonitrotetrazolium chloride
LPS	Lipopolysaccharides
MA	Malt agar
MCF-7	Human breast adenocarcinoma
MDA	Malonaldehyde
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MHB	Mueller-Hinton agar
MUFA	Monounsaturated Fatty Acids
MS	Mass spectrometer
NCI-H460	Lung cancer cell line
NO	Nitric Oxide
OxHLIA	Oxidative Haemolysis Inhibition Assay
PUFA	Polyunsaturated Fatty Acids
rpm	Revolutions per minute
RSA	Radical Scavenging Activity
SFA	Saturated Fatty Acids
SRB	Sulforhodamine B
ST	Storage time

TBARS	Thiobarbituric Acid Reactive Substances
UV	Ultraviolet Radiation
v/v	Volume per volume
w/v	Weight per volume

1 Introduction

Food intake is no longer seen as just a form of hunger satiation, to be associated with the intake of nutrients and bioactive compounds, with a direct influence on consumer health and well-being (Kovačević et al., 2020). In this sense, consumers are increasingly aware of their food choices and there is a greater concern with the analysis of labels, favoring products designated as healthier and more natural (Liu, 2013).

In this sense, there is a concern on the part of the food industry to follow new consumer trends and, in the launch of this type of products on the market (Kovačević et al., 2020). The incorporation of natural ingredients with functional and bioactive properties has been tested. Nature presents several natural matrices that have been studied and tested as promising sources of natural ingredients to be used by the industry (Lima et al., 2017).

There is a high amount of bio-waste discarded annually by the food industry and commercialization, which has been raising a great concern not only economic but also environmental. This type of waste has been characterized as having a very interesting nutritional and chemical composition, in addition to highlighting the presence of bioactive compounds of high interest (Lima et al., 2017). Recent statistics showed that European food processing units might generate approximately 100 Mt of waste and by-products each year, mostly comprising the production of drinks (26%), dairy and ice cream (21.3%), and fruit- and vegetable-derived products (14.8%) (Maric, 2018).

Around 70 % to 80 % of the total apple produced is destined for marketing in fresh, and the apple considered to be of inferior quality is destined for the agri-food industry, for processing. Fruits that present characteristics that do not fit the classification and selection standards, such as, for example, low caliber, non-uniform colour, scars caused by insects, birds or hail, injuries resulting from cultural methodologies, such as harvesting, inadequate transport, symptoms disease or physiological problems, are rejected for marketing (Codex Standard for Apples, 2010). Initially, these fruits were intended for animal feed, but with the increase in raw material, the food industries started to process them, as a form of economic valorization (Paganini et al., 2004).

The apple and its residues appear as rich sources of antioxidants that can play a role in reducing the risk of several diseases related to oxidative stress, for example, coronary heart

disease, damage to the immune system, asthma, and diabetes, which has aroused great interest by the scientific community and the industry (Zhang et al.,2016).

1.1 AGRI-FOOD WASTE AS SOURCES OF BIOACTIVE COMPOUNDS

In the food industry, waste is all unused or unconsumed parts of a fruit, vegetable, or other food. Its disposal can be justified by the morphological characteristics not being in accordance with the standardized ones, inadequate handling or simply discarding parts of the elements without use. However, the amount and type of waste may vary by industry, morphological components, environmental factors and may include leaves, roots, tubers, skin, pulp and/or seeds. Many fruits and vegetables generate about 25 to 30% of waste, which is discarded without any use, creating serious environmental and health problems (Parfitt et al., 2010).

Food and Agricultural Organization (FAO, 2020) reports have shown that higher loss rates occur for roots, tubers, and oilseeds (25%) followed by fruits and vegetables (21 %). Cereals and pulses only lose up to 8%.

According to data presented by the FAO, approximately 30% of food produced for human consumption worldwide is lost or wasted each year. This equates to millions of tons of wasted food and large economic costs and negative environmental impacts. **Figure 1** shows the loss of food during processing in Europe, America and Oceania and Asia. In this way, the use of these bio-wastes becomes an emerging issue all over the world.

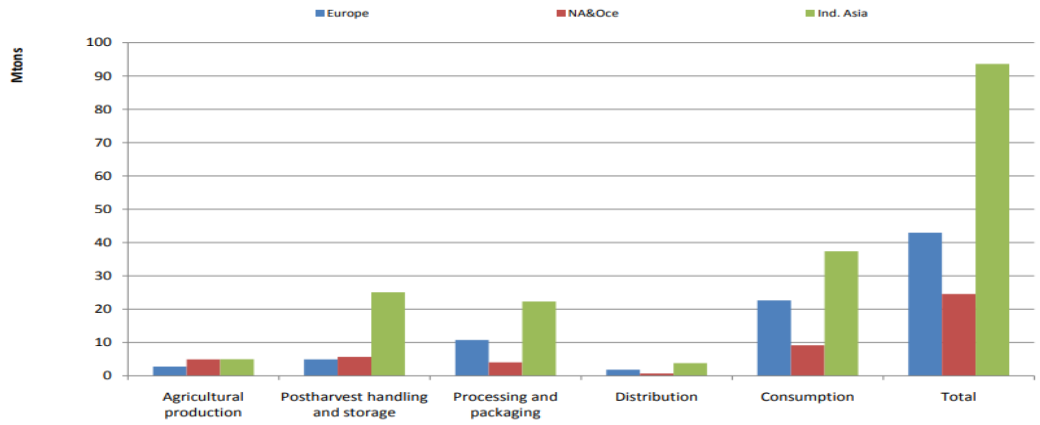

Figure 1. Global Food Losses and Food Waste *(Jenny Gustavsson, Christel Cederberg & Ulf Sonesson SIK – The Swedish Institute for Food and Biotechnology Save Food Congress, Düsseldorf 16 May 2011)*

Food losses can be reduced, but not completely avoided. These residues are enormous sources of bioactive molecules namely, polyphenols, dietary fiber, carbohydrates, and proteins, with wide applications (Ng et al., 2020). Many of these bioactive compounds have antioxidant, antidiabetic and anti-inflammatory properties, cardiovascular protection, and anticancer activities, thus being able to act as health promoters. Thus, these compounds have been evaluated to validate their potential as functional foods (Kumar, 2015), nutraceutical (Gupta et al., 2017), pharmaceutical (Baiano, 2014) and beauty care products (Ribeiro et al., 2013).

The literature highlights the high content of different phenolic compounds in fruit and vegetable residues, justifying the excellent bioactive potential with a view to its industrial application.

1.1.1 **The current problem of waste in the food industry**

In recent years, waste recovery has gained prominence as an alternative option to waste disposal in landfills, since it takes some time to be mineralized, which causes an accumulation of organic matter (Gonçalves et al., 2016). The recovery of waste is fundamental to the concept of circular economy, which has currently gained considerable social, economic, environmental, and political relevance (Mayer et al., 2019). Minimizing waste or making it less harmful depends on actions throughout the product lifecycle. In this way, the use of bio-waste is seen as an environmentally and ecologically correct practice, in addition to providing new products capable of bringing benefits to consumer health.

The huge quantity of products generated in the market, the lack of technological innovations in the processing of raw materials and infrastructure, inadequate food safety protocols, overcooking, unwanted sizes and weights in food, the defects of the packaging and labeling, are aspects that contribute to the food waste generation during the food supply chain, also with bad cultural habits for the purchases and consumption (De Moraes, 2020).

These residues are characterized by high moisture content, high biological instability, and high organic load, which in turn promotes microbial activity and is therefore difficult to handle. Inadequate practices for disposing of these residues cause environmental problems, such as toxicity to aquatic life, contamination of surface and underground waters, alteration of soil quality, emit greenhouse gases, in addition to attracting vectors such as insects and rodents, among others. Thus, several treatment alternatives have been studied in order to improve this

environmental scenario and also obtain some economic benefit through the recovery of waste (Nanda et al., 2016).

Food losses in industrialized countries are as high as in developing countries, but in developing countries more than 40% of food loss occurs at the postharvest and processing levels, while in industrialized countries, more than 40% of food loss occurs in retail and consumer settings. levels. Food waste at the consumer level in industrialized countries is almost as high as total net food production in sub-Saharan Africa (Pap & Myllykoski, 2014). **Table 1** highlights wastage in different food sectors, with the stages of the highest wastage and the reasons of wastage.

Table 1. Food loss in different sectors (Gustavsson et al., 2011).

Food sector	Part of initial produce lost	Stage of highest food loss and percentage of loss at that stage		Reason for wastage
Cereals	20-35%	Consumption	2-25%	Wasted by consumers
Roots and tubers	32-60%	Agricultural production	5-20%	Postharvest crop grading due to quality standards set by retailers
Oily crops and pulses	18-30%	Agricultural production	6-12%	Lost during harvest
Fruits and vegetables	35-55%	Consumption	2-15%	Wasted by consumers
		Agricultural production	10-20%	Postharvest fruit and vegetable
Meat and meat products	20-28%	Consumption	2-10%	Wasted by consumers
		Animal production	2-15%	Animal mortality during breeding and transportation
Fish and seafood	30-50%	Fisheries	5-15%	Discard of marine catches
		Consumption	2-25%	Wasted by consumers
Dairy products	10-25%	Consumption	2-15%	Wasted by consumers

Food waste can be classified into three categories: avoidable food waste, unavoidable food waste and possibly avoidable food waste. Avoidable waste refers to food that could have been eaten at some point prior to being thrown away. Unavoidable food waste refers to the fraction of food that is not usually eaten (banana peels and chicken bones). Possibly avoidable food waste refers to food that is eaten in some situations but not others (potato skins) (Papargyropoulou et al., 2014).

The use of residues generated in the processing of fruits and vegetables as a source of bioactive compounds is an alternative pointed out by several authors since they are excellent

sources of pigments, phenolic compounds, dietary fibers, sugar derivatives, organic acids, minerals, dyes, among other components characterized by having several health benefits (Sagar et al., 2018).

1.1.2 **The particular case of apples bio residues**

The apple (*Malus domestica* Borkh) is probably the oldest fruit known to man and preferred by millions of people in the world. The apple originates from West Asia, having spread to all regions that have soil and climate conditions conducive to its production. There are thought to be over 7500 varieties of apple. The apple tree blooms in spring and its fruits are harvested in late summer and early autumn in the Northern Hemisphere. Apples are the fourth most produced fruit in the world after bananas, oranges, and grapes Worldwide, apple production is estimated at 67 million tons (about 56% is produced in Asia, 26% in Europe, 15% in America, 2% in Africa and 1% in Oceania). In Portugal, the estimated average production is 265,000 tons/year, that is, 0.4% of world production (Mahawar et al., 2012).

Apples are considered a good source of nutrition. They are major suppliers of water (82.9%), vitamins (particularly vitamin C), mineral salts (especially potassium), fiber (2.1%), fermentable sugars, pectin and a large amount of phytochemicals (Barrett et al., 2010). It is a fruit that does not contain fat, is an excellent source of fiber and does not contain cholesterol. In addition to being rich in phytonutrients and fiber, an apple is low in calories, making it a great food. Apples constitute an essential part of the human diet as they are an important source of monosaccharides, minerals, dietary fiber, vitamins, and phenolic compounds are known to act as natural antioxidants (Wu et al., 2007). Apple is produced on a large scale due to its beneficial effects of preventing chronic cardiovascular disease, respiratory and pulmonary disorders, diabetes, obesity, or cancer (Tu et al, 2017).

Fruits considered of low caliber or with some defects are considered unsuitable to be sold to the consumer, so they are often discarded as waste. Likewise, peels, pits and seeds are discarded when making processed products from this fruit. Fruit pulp and apple pomace are also residues resulting from processing. Therefore, this industrial activity generates a large number of underused by-products, which can be expensive and complex to remove. Adding value to these materials might produce economic benefits, while slightly reducing the huge volume of by-products demanding suitable disposal strategies (Barreira, et al. 2019).

Recent data indicate that apple has a very high production volume, around 87 million tons in 2019 (FAOSTAT, 2019). In Portugal, the productive sector recorded in 2019 the highest value of the last 30 years, with around 354 thousand tons, which translates into a turnover of around 75 million euros (Statistics Portugal, 2019). Apples are used for fresh consumption which accounts for 70%-75% while the rest is processed into various value-added products including juices, wines, jams and produce (Bortolini et al., 2020). However, apple juice remains the most in-demand apple product, accounting for 65% of total apple processing. Collectively, nearly 75% of the fresh apple weight is thought to be extracted as juice during juice production, and the remainder is collected as food waste, the so-called pulp (Fengzhi et al, 2020). Considering the worldwide mass production of juice, a total of several million metric tons of the pomace is estimated to be generated every year (Kammerer et al., 2014). **Figure 2** presents a scheme that illustrates the waste generated from the apple processing

Apple pomace is the residual material that remains after juice and cider pressing and represents up to 25% of the fresh fruit weight. Since apple pomace is generated in vast quantities and contains a large fraction of water, it poses storage problems and requires immediate treatments to prevent putrefaction. An alternative of great environmental interest is its transformation into value added commodities, thus reducing the volume of waste (WAPA, 2016).

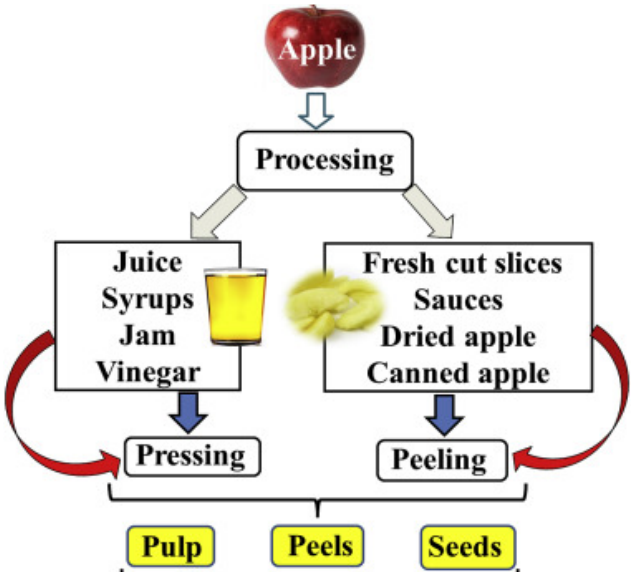

Figure 2. *Generation of apple pomace during the processing of apples to produce different by-products* (Nile et al., 2019).

1.1.3 **Apple bio-waste as source of bioactive compounds**

Fruit and vegetable residues are rich sources of phytochemicals and have been studied for the extraction of phenolic compounds, dietary fibers, and other bioactive compounds. In most fruits and vegetables, only the pulp is consumed, but studies have revealed that significant amounts of phytochemicals and essential nutrients are present in the seeds, peels, and other components of uneaten fruits and vegetables (Rudra et al., 2015). For example, the rinds of lemons, kiwis, grapes, oranges and others, and the seeds of avocados, jackfruit, longans and mangoes contain more phenolic concentrations than those found in the respective pulps (Oliveira et al., 2015; Lima et al., 2017; Dias et al., 2020).

As these residues are generated in large quantities by the processing industry, they become a good alternative for obtaining extracts rich in bioactive compounds. For example, extracts of by-products (peels and seeds) generated from processing grapes for juice or wine production have been shown to be excellent sources of various phenolic compounds (Moro et al., 2021). In a similar study, (Lima et al., 2017) evaluated the total phenolic content and antioxidant activity of pineapple residues derived from industrial processing, and the results showed that the extract contains amounts of protein and lipids similar to those present in the pulps and, higher fiber content. In addition, the juice extracted from the peel had a low content of carbohydrates and calories, and a high content of phenolic compounds, which justifies the high antioxidant capacity. Likewise, a study on the peel of different kiwi varieties revealed that they showed higher antioxidant activity compared to pulps, emphasizing their potential for application in different industrial areas (Dias et al., 2020).

Apple pomace is the main by-product of apple cider and juice processing industries and accounts for about 25% of the original fruit mass at 85% moisture content 66.4–78.2% moisture and 9.5–22.0% carbohydrates (Sun et al. 2007). apple pomace contains 26.4% dry matter, 4.0% proteins, 3.6% sugars, 6.8% cellulose, 0.38% ash, 0.42% acid and calcium, small amounts of minerals, and vitamins (Cargnin and Gnoatto,2017). These by-products are the highest source of dietary antioxidants, after oranges, it is one of the main dietary sources of antioxidants, especially phenolic compounds, such as flavonoids and phenolic acid. Other constituents such as terpenoids, have been associated with antioxidant, antibacterial, and antitumor properties (Grigoras, 2013; Farneti, 2015).

The chemical composition of apples is strongly influenced by the variety/cultivate, production conditions (light, temperature, etc.), cultural techniques, harvest date, maturation

index, storage conditions, post-harvest handling of the fruit, among other factors (Barrett et al., 2010).

Apples are excellent sources of vitamins, especially vitamin C (ascorbic acid), vitamin E (α-tocopherol) and the B vitamins (thiamine, riboflavin, B6, niacin and folate). These fruits are also an important source of β-carotene, a precursor of vitamin A. The levels of vitamin C and vitamin A decrease during the storage period. Vitamin C is degraded relatively quickly by exposure to heat, light, and oxygen (Barrett et al., 2010).

Ascorbic acid, together with phenolic compounds, is an important source of antioxidants in apples (Gliszczynska-Swiglo & Tyrakowska, 2003). Antioxidants can play a significant role in maintaining fruit integrity during apple development on the tree and during storage (Davey et al., 2007).

Phenolic compounds contribute to the colour and flavor of the fruits. Some of these compounds, in addition to influencing the taste, impart bitterness and astringency to the products in which they are present. These compounds can be classified into flavonoids (anthocyanins, flavonols, flavones, flavonones, betacyanins), isoflavones, phenolic acids (hydroxybenzoic acids, hydroxycinnamic acids) and stilbenes. The main compounds present in the polyphenolic fraction of apples were catechins and proanthocyanidins followed by hydroxycinnamates, flavonols, dihydrochalcones, and anthocyanins. However, the number of individual compounds varies up to 30% from one year to another in the same cultivar (Pascoalino et al., 2021). The phenolic compounds present in apples are chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, (+) catechin, (-) epicatechin, procyanidins (B1, B2, C1 trimer), rutin, quercetins, phloridizin and phloretin (Karaman et al., 2010). **Figure 3** shows the structure of the most abundant phenolic compounds present in apples.

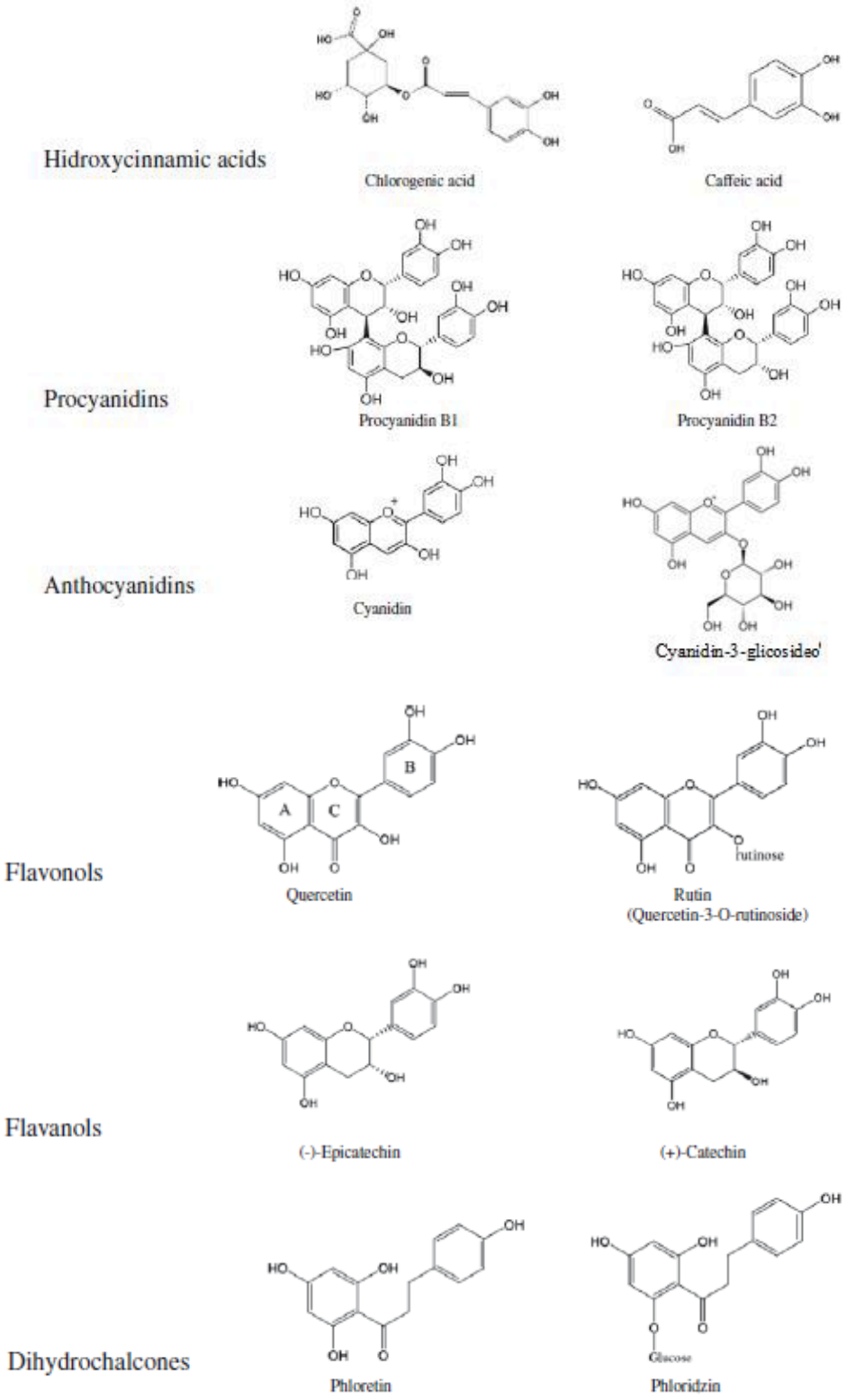

Figure 3. Structure of phenolic compounds present in apple

(Pascoalino et al.,2021)

Apple pomace, which includes soft tissues, core, stems, seeds, and peels, is a good source of polyphenols such as chlorogenic acid, hydroxycinnamates, quercetin and catechin.

The colour of the apples can vary between green, yellow, and red (**Figure 4**). Colour is a function of natural pigments present in fruits and vegetables, including fat-soluble dyes such as chlorophylls (green), carotenoids (yellow, orange and red), water-soluble dyes such as anthocyanins (red, blue) and flavonoids (yellow) (Barrett et al., 2010). In apples of red varieties, the pigment responsible for the colour is an anthocyanin, mainly cyanidin-3-galactoside (Iglesias et al., 2008). Colour can be modified at maturation, during fruit ripening, fruit handling and storage conditions.

Figure 4. Colour varieties of apples
(Pascoalino et al.,2021)

The rich phenolic composition present in apples and their residues has been arousing high interest in the industry since several studies have associated this composition with the excellent bioactive properties (namely antioxidant and antimicrobial activity) proven in these natural matrices (Miklasínska-Majdanik, 2018; Andrade, 2019). Procyanidins and florethine are polyphenols present in apple that demonstrate anti-inflammatory activities and can function as inhibitors of the expression of pro-inflammatory genes based on the transcription. Apple pomace is also characterized by a high content of fibers (cellulose, hemicellulose, pectin, β-glucans, gums, and lignin) that play an important role in human health, diets rich in fibers have been associated with the prevention, reduction, and treatment of several diseases, including diverticulitis, coronary heart disease, colon cancer, obesity, diabetes, asthma, and pulmonary disease (Vendruscolo et al 2008).

1.2 FUNCTIONAL AND DIETARY FOODS AND THEIR GROWING CONSUMER DEMAND

Changes in lifestyle, stress, lack of physical activity, and inadequate nutrition have been identified as the main reasons that led to the spread of many diseases, causing about 41 million deaths annually, which represent 71% of all deaths. Cardiovascular diseases pose the greatest risk (17.9 million deaths) followed by diseases related to cancer (9.0 million), respiratory diseases (3.9 million), and diabetes (1.6 million) (Plasek et al., 2019).

Consumers are increasingly aware of the direct relationship between food and their health and well-being, so they have been making healthier, natural, and functional food choices. In this way, the food industry has been looking for new natural ingredients with bioactive properties to develop new, innovative, healthy, and low-cost products that meet consumer expectations (Bigliardi & Galati, 2013).

The hypothesis of proper nutrition has recently been scientifically proven to be one of the main factors behind an individual's well-being. The consumer has become more aware of the concept of "a human being is what he eats" and has begun to search for alternative and varied answers depending on age and gender in terms of dietetics. Unfortunately, the modern diet is rich only in what seems “outwardly” and rich in the sense that on the one hand the amount of food increases, and on the other hand the quality of nutrition decreases (Jahan, 2015).

Although there is no universally accepted definition for functional foods, it is assumed that a food acquires the status of a functional food when, in addition to its basic nutritional function, it guarantees to have beneficial physiological effects on health, whether specifically in reducing the risk of disease development or improvement of a health situation (Plasek et al., 2019). This type of food arouses growing interest since, in addition to playing a very important role as a health promoter, it has an important economic impact with a direct influence on reducing health costs as well as improving the quality of life (Carocho et al., 2014).

Functional foods are complex mixtures that can intercept the active compound, modulate its release or inhibit its activity. In this way, the food matrix, both in its raw state and after cooking or storage, can have a significant influence on the activity or release of the main components. Thus, a crucial step towards the success of functional foods is the

development of suitable food vehicles to maintain the active form until the moment of consumption, and delivery to the desired target site in the body (Betoret et al., 2011).

There are several natural matrices that have been studied and identified as excellent sources of bioactive compounds. The main bioactive compounds and functional ingredients added to foods are probiotics, prebiotics, dietary fiber, phytosterols, carotenoids, phenolic compounds, and fatty acids.

A functional food can be obtained through different ways: eliminating a component that causes harmful effects when consumed (e.g.: allergenic proteins); increasing the concentration of a natural component present in the food up to a point where it can induce beneficial effects (e.g.: fortification with a micronutrient to increase daily intake over recommended); addition of a component which is not normally present in many foods and which is not necessary as a macro- or micronutrient but for whose beneficial effects it has been used (e.g.: non-vitamin anti-oxidants i.e. the hydrophilic polyphenols plus the their glycosides or prebiotic fructan); replacing a component, usually macro-nutrient (fatty acids) that is excessive with a component with beneficial effects (modified starch); increasing the bioavailability or stability of a recognized component for its functional effects or reducing the potential risk of the disease (Vasileva, 2015; Righi,2018).

Most of the early developments of functional foods were those fortified with vitamins and/or minerals such as vitamin C, vitamin E, folic acid, zinc, iron and calcium. Then to foods fortified with multiple micronutrients such as omega-3 fatty acids, phytosterols, and soluble fiber to promote good health (Siró, et al 2008).

Dairy, pastry, bakery, and baby food products emerge as the functional food markets with the greatest evolution and investment (Bigliardi & Galati, 2013). Currently, some food products with beneficial health functions are marketed. Some successful examples are: Danone® branded probiotic liquid yogurt, Actimel® containing *Lactobacillus casei* Imunitass® cultures or Activia® solid yogurt from the same brand containing Bifidus ActiRegularis®. Bakery products provide ideal matrix by which functionality can be delivered to the consumer in an acceptable food. In 2003, Unilever® innovated the bakery sector by introducing a white bread called *Blue Band Goede Start*, which was the first white bread containing the nutritional elements normally available in brown bread including fibers, vitamins B1, B3 and B6; iron; zinc; inulin, a starch that comes from wheat.

1.2.1 **Alternative Flours with health benefits**

Bread is a food product much appreciated worldwide and is recognized as a fundamental component of the Mediterranean diet. Flour is the basic ingredient in the preparation of breads, so its quality is essential to guarantee a quality product (Babarinde et al., 2020).

Currently, the bakery industry has been looking for new ingredients for the preparation of bread labeled as healthier. In this way, they intend to meet current consumer expectations and circumvent the idea that bread is an unhealthy food. Wheat is a natural source of vitamins like vitamin B, vitamin E, iron, and zinc. However, many of these essential components can be lost during milling and refining, making the final flour product less nutritious (Pourafshar et al., 2010). Bakeries have been manufacturing a wide variety of bakery products containing bioactive components such as dietary fiber, antioxidants, and phenolic compounds (Gomez & Martinez, 2018; Spina et al., 2019).

Wheat flour is the most popular flour in the baking industry and despite being nutritionally poor, it guarantees improved texture and appearance (Dalal & Bobade, 2018). Although there are wheat flours with different elements and nutritional qualities, there are inadequacies and specific challenges for which it must be complemented, enriched or even replaced by other flours. Deficiencies of certain micronutrients, such as vitamins and minerals, or problems with wheat gluten, which can sometimes cause a variety of sensitivities and disorders in people, are examples of these problems. In this way, the nutritional content of bakery products can be improved by totally or partially replacing wheat flour with alternative flours, which meets consumers' desires for nutritious and diversified foods (Hadnađev et al., 2011).

Almonds stand out for their various health benefits, namely in aiding fat loss and controlling diabetes. In this way, some pastry products, namely cakes and cookies, have been made with this flour, guaranteeing products with low carbohydrate and salt content and high potassium content, being labeled as suitable for diabetics (Richardson et al., 2009).

Almonds are rich in vitamins, such as vitamin E, as well as antioxidants. As almond flour does not contain gluten, it can be used by people with celiac disease. Almonds can be eaten alone or used in breads, pastries, desserts and spreads, making them an ideal substitute for peanuts for anyone who is allergic to peanuts (Richardson et al., 2009)

Potato flour is another alternative flour created from boiled, dried and ground potatoes that has been tested by the food industry, namely cakes, cookies and pasta, but also as a thickener for soups and sauces (Daley, 2001). As potato starch has a larger granule size than

other starches, this flour is considered gluten-free and therefore suitable for consumption by gluten-intolerant consumers (Shepherd & Gibson, 2006). Rice flour, the result of crushed rice and reduced to a very fine powder, is also considered a gluten-free ingredient and widely used in the production of products suitable for this type of consumer (Pourafshar, 2010).

In turn, rye is cereal rich in physiologically active compounds, including antioxidants, and is suggested as part of a healthy diet (Zielinski et al., 2007). Rye flour comes in two varieties: dark and whole meal, the former being highlighted with the richest in protein, vitamins, and minerals (Daley, 2001).

After wheat and rice, corn is the most important cereal in the world, providing nutrition for people and animals (FAO, 2020). Corn is used to make a wide variety of products, including corn kernels of various sizes, cornmeal, cornmeal, and flaked kernels. Corn flour is prepared from dry corn that comes in yellow, white, and even red colours and has been used by the food industry in the preparation of various products (Olexova et al, 2006).

Given the current concerns about finding solutions for the recovery of bio-waste from the agro-fruit industry discarded annually in worrying quantities and, with negative impacts on the economy and the environment, its use in the industry has been explored due to its interesting bioactive content. Additionally, several studies have highlighted the bark and seeds as sources of bioactive compounds. As a result, they are promising and inexpensive substrates for the extraction of phytochemicals with diversified use, both in the food industry and in other industries, such as pharmaceuticals and cosmetics. The most common reuse of by-products generated by the agri-food industry is as animal feed or as fertilizer. Other ways found have been the incorporation of these by-products in food, with the purpose of developing new products with attractive quality to the consumer and thus also helping in the environmental impact (Elvas, 2016).

An example is banana flour, a by-product of green bananas, which has high starch content and has been noted for having significant therapeutic effects, especially in situations of gastrointestinal infection. This flour has been incorporated in formulas for new-borns and in bakery products (Martinez et al., 2009).

1.2.2 Potential for industrial application of apple flour obtained from bio waste

Although there is no universally accepted definition for functional foods, it is assumed that a food acquires the status of a functional food when, in addition to its basic nutritional function, it guarantees to have beneficial physiological effects on health, whether specifically in reducing the risk of disease development or improvement of a health situation. This type of food arouses growing interest since, in addition to playing a very important role as a health promoter, it has an important economic impact since it will have a direct influence on reducing health costs as well as improving the quality of food life (Carocho et al., 2014).

Functional foods are complex mixtures that can intercept the active compound, modulate its release, or inhibit its activity. In this way, the food matrix, both in its raw state and after cooking or storage, can have a significant influence on the activity or release of the main components. Thus, a crucial step towards the success of functional foods is the development of suitable food vehicles to maintain the active form until the moment of consumption, and delivery to the desired target site in the body (Betoret et al., 2011).

Thus, it is consensual that the development of functional foods is essential to offer consumers alternative and healthier food options. Fruit and vegetable residues are considered valuable sources of dietary fiber and antioxidants with great potential for application in the food industry, namely in the development of functional foods (Díaz-Vela, et al 2015).

The fruit industry annually produces tons of bio-waste throughout the different processing stages. These residues, namely, husks and seeds, can be used in different ways, one of them is in the manufacture of flour, which can be used in several recipes, as it is rich in nutrients such as fiber, minerals, and vitamins (Fontes et al., 2014). Apple flour is an example of flour rich in soluble and insoluble fiber characterized by promoting a better quality of life by stimulating the intestinal flora (Bingham et al, 2003; Aune et al, 2011).

Apple flour is characterized by containing 100, 20, 15 and 12.5 times higher dietary fiber content than flour obtained from rice, buckwheat, corn, and wheat flour, respectively. In this way, it has been explored for use in confectionery and bakery products in order to compensate for the low dietary fiber content of conventional products. In addition, the potassium content of apple flour is 5 times higher than that of rice flour and 3 times higher than that of corn flour. Lower levels of potassium in a diet have been found to be associated with a higher risk of hypertension, type 2 diabetes. Additionally, apple flour is characterized by being

gluten-free. It is also important to note that apple flour has a caloric value of 15 to 20% less than wheat flour (Zlatanovic, 2019).

Another important fact that has made apple flour stand out from others is the fact that its content of phenolic compounds is 60, 50, 8 and 1.5 times higher than wheat, rice, corn, and buckwheat flour, respectively. This content has been pointed out as responsible for the excellent antioxidant activity presented by apple flour. For all these reasons, the addition of apple flour to foods has represented a great challenge for the food industry in the development of new alternative and functional products with beneficial characteristics for the consumer (Zlatanovic, 2019).

OBJECTIVES

The agri-food industries, which include producing companies and apple processing industries, have increased their growth worldwide. This increase has consequently generated millions of tons of agro-industrial waste with great economic and environmental impact. In addition to this concern with the use of bio-residues from fruits and vegetables, there is a need to valorize residues that, several studies classify as a good source of functional compounds and, consequently, their application in the food industry appears as very promising. In this sense, this work intends to highlight the potential of industrial apple bio-waste as a source of functional compounds through the elaboration of apple flour as a new added-value food ingredient for the nutritional fortification of a bakery product (**Figure 5**).

The specific objectives of this work were:

- Development of an apple flour from the industrial apple bio-waste;
- Nutritional characterization (fats, ash, proteins, carbohydrates and energy) of the flour;
- Chemical characterization (content in sugars, fatty acids and organic acids) of apple flour;
- Characterization of the flour's phenolic profile;
- Study of bioactivities (antioxidant, antimicrobial, cytotoxicity and anti-inflammatory) of apple flour;
- Assessment of the potential for incorporating apple flour in a pastry product to replace traditional flour.

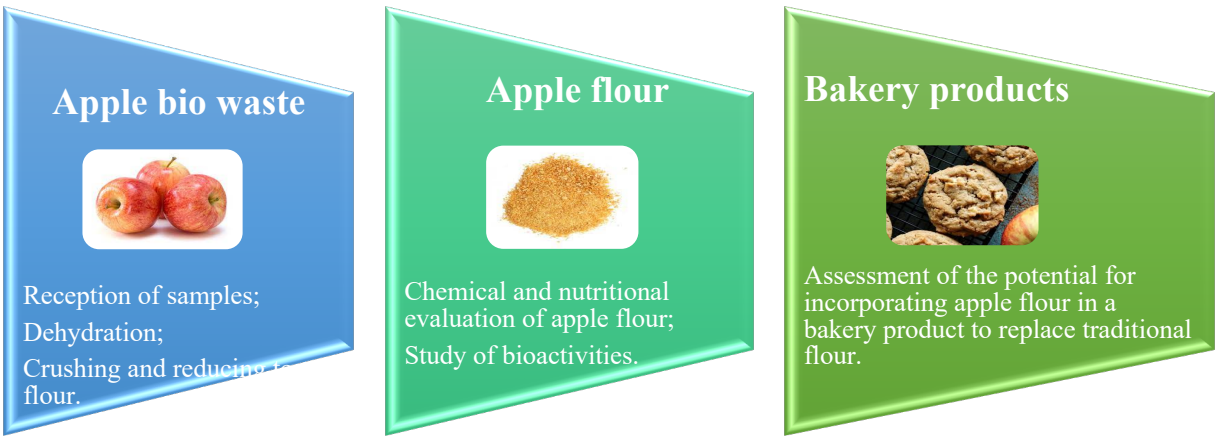

Figure 5. Schematic representation of the objective of this work. (Author authorship)

2 Materials and Methods

2.1 Apple flour preparation

The Fuji variety apples were kindly provided by the Campotec company, based in Torres Vedras, Portugal. After reception the apples were washed with water. The apples were cut into slices and the cores and seeds were removed. The apple slices were placed to dry in the oven at 50°C for 48 hours and then crushed and reduced to flour. Subsequently, the flour was stored in a cool and dry place, protected from light, for further analysis (**Figure 6**).

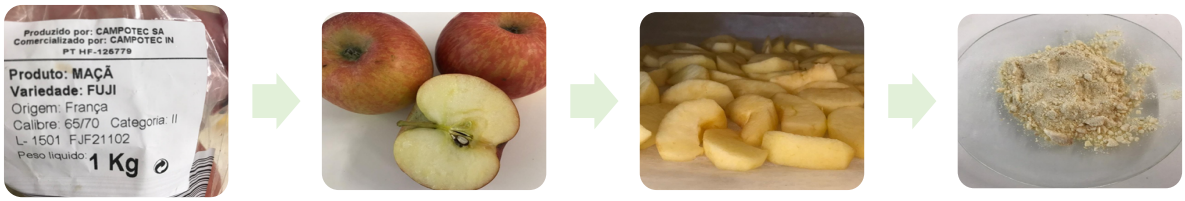

Figure 6. The process for obtaining the apple flour (Author authorship).

2.2 Standard and reagents

HPLC grade n-hexane, 99% acetonitrile and 99.98% ethyl acetate solvents were purchased from Fisher Scientific (Lisbon, Portugal) and analytical grade methanol solvent was purchased from Pronalab (Lisbon, Portugal). The water was treated prior to use by the Milli-Q-Water purification system (TGI Pure Water Systems, Greenville, SC, USA).

The standard blend with 37 fatty acid methyl esters (FAME) (C4-C24; standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as well as other individual fatty acid isomers, sugar standards ((D(-)-fructose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose and D(+)-raffinose pentahydrate), organic acid standards (L(+)-ascorbic acid ; citric acid; malic acid; oxalic acid; shikimic acid; succinic acid; fumaric acid and quinic acid).

Fetal bovine serum (FBS), L-glutamine, Hank's saline solution (HBSS), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) and DMEM medium (medium for animal cells (Dulbecco Modified Eagle)) were purchased from Hyclone (Logan,

Utah, USA). Acetic acid, ellipticin, sulforodamine B (SRB), trichloroacetic acid (TCA) and Tris were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Mueller-Hinton agar (MHB) was obtained from Biolab® (Hungary). The compound p-Iodonitrotetrazolium chloride (INT) was purchased from Panreac Applichem (Barcelona, Spain).

2.3 Determination of the nutritional value of apple flour

The macronutrient composition of the Apple flour was determined using official food analysis methodologies (AOAC, 2016).

The lipid content was determined by extracting a known mass apple flour (3 g) with petroleum ether at 80 °C for about 7-8 hours using a Soxhlet apparatus (**Figure 7**), and then the fraction was evaporated. Volatile, the resulting extract was then placed in an oven until constant weight, in order to determine the fat content (AOAC 989.05).

Figure 7. Soxhlet extraction (Author authorship).

The ash content was determined by incineration in a muffle (**Figure 8**) at 550 ± 15°C for 12 hours (AOAC 935.42).

Figure 8. Crucibles used for the evaluation of ash contente (Author authorship).

The protein content (N×6.25) was obtained by the macro-Kjeldahl method (AOAC 991.02), (AOAC, 2016), based on the amount of nitrogen present in the sample and on the destruction of all organic matter by the addition of a strong acid (sulfuric acid) which retains nitrogen in the form of $(NH_4)_2SO_4$. Addition of NaOH releases the nitrogen as NH_3 which is collected by steam distillation in a 0.1N H_2SO_4 solution; then a titration is carried out with 0.1N NaOH, using methyl red as an indicator to calculate the amount of nitrogen.

Carbohydrates were determined by difference and total energy was determined according to **Equation 1**:

$$\text{Energetic value (kcal)} = 4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g lipids}).$$

Equation 1. Equation to determine total energy.

2.4 Determination of chemical composition of apple flour

2.4.1 ***Free sugars***

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) (Barros et al. 2013). To the sample (1.0 g) an internal standard was added - melezitose (PI, 25 mg/mL), and the extraction was carried out with 40 mL of an ethanol/water solution (80:20, v/v), for 1h 30 min. The resulting suspension was

centrifuged (refrigerated centrifuge: Centurion K24OR, West Sussex, UK) at 15,000g for 10 min and the ethanol fraction was evaporated (rotary evaporator Büchi R-210, Flawil, Switzerland) at 35°C under reduced pressure. The aqueous fraction was defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL and filtered through nylon filters (Whatman 0.2 µm) into an amber vial for further analysis (**Figure 9**).

Figure 9. Tubes used for the evaluation of free sugar (Author authorship).

The HPLC equipment consisted of an integrated system with a pump (Knauer, Smartline 1000 system, Berlin, Germany), a degassing system (Smartline manager 5000), an autosampler (AS-2057 Jasco, Easton, MD, USA) and a detector. refractive index (RI) (Knauer Smartline 2300).

Data analysis was performed using Clarity 2.4 software (DataApex) and chromatographic separation was performed with a Eurospher 100-5 NH2 column (4.6 x 250 mm, 5 µm, Knauer) operating at 35 °C (7971 R Grace oven). The mobile phase used was acetonitrile/deionized water, 70:30 (v/v), at a flow rate of 1 ml/min and an injection volume of 10 µl.

The identification of compounds was performed through chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were expressed in g per 100 g of dry weight (dw).

2.4.2 ***Organic acids***

Organic acids were determined using high performance liquid chromatography coupled to a diode detector (UFLC-DAD). Samples (approximately 1 g) were extracted by steeping with 25 ml of metaphosphoric acid (25°C at 150 rpm) for 20 min. Subsequently the mixture was centrifuged (4000g, 10 min) and then filtered through nylon filters (Whatman, 0.2 μm) into a 1.5 mL vial (Barros et al., 2013).

Analysis was performed using a UFLC Shimadzu 20A series (Shimadzu Corporation, Kyoto, Japan). Separation was performed on a SphereClone reversed-phase C18 column (250 mm x 4.6 mm i.d., 5 μm, Phenomenex, Torrance, CA, USA) thermostated at 35°C and eluted with sulfuric acid (3.6 mM), using a flow rate of 0.8 mL/min, and an injection volume of 20 μL.

The detection was performed using a DAD, using 215 and 245 nm as wavelengths. The organic acids found were quantified by comparing the area of peaks recorded at 215 nm and 245 nm (for ascorbic acid) with calibration curves obtained from commercial standards for each compound. The results were expressed in g per 100 g of dry weight (dw).

2.4.3 ***Fatty acids***

The determination of the fatty acid profile was performed using a gas chromatography system coupled to a flame ionization detector (GC-FID) following a procedure previously described by Barros et al. (2013). The mass obtained by Soxhlet extraction was mixed with 5 ml of methanol: sulfuric acid: toluene 2:1:1 (v/v/v), for at least 12 h, in a bath (Julabo, SW22; Seelbach, Germany) at 50°C and 160 rpm; then 3 mL of deionized water were added to obtain phase separation. The FAME was recovered with 3 mL of ethyl ether, after vortexing, the sample was recovered in a flask with Teflon and anhydrous sodium sulfate was placed in order to eliminate the water. Subsequently, the sample was filtered with a 0.2 μm nylon filter (Whatman) into a 1.5 mL vial.

The fatty acid profile was analyzed using a GC system (DANI 1000) equipped with a split/splitless inlet, flame ionization detector (FID) and a Macherey Nagel column (30 m x 0.32 mm i.d. x 0.25 μm d*f*). The oven temperature program was: initial column temperature of 50°C, held for 2 min, then a 30°C/min rise to 125°C, 5°C/min rise to 160°C, 20°C/min rise to 180°C, 3°C/min rise to 200°C, 20°C/min rise to 220°C and hold for 15 min. The flow of hydrogen

(carrier gas) was 4.0 mL/min (0.61 bar), measured at 50°C. Split injection (1:40) was performed at 250 °C. For each analysis, 0.1 µL of the sample was injected and the identification of fatty acids was made by comparing the relative retention times of the FAME peaks of the samples with the standards. Results were recorded and processed using CSW Software 1.7 (DataApex 1.7, Prague, Czech Republic) and expressed as a relative percentage (%) of each fatty acid.

2.4.4 ***Determination of the profile of phenolic compounds***

Approximately 1 g of apple flour was weighed and 30 mL of an ethanol/water solution (80:20, v/v) were added, leaving the mixture to macerate for 1 hour, with stirring. After the maceration process, the supernatant was filtered using filter paper (Whatman Nº4) and the residue was re-extracted by adding again 30 mL of the same solvent mixture. The ethanolic fraction of the extract was evaporated at 40 °C (Büchi R-210, Flawil, Switzerland), resulting in an aqueous fraction that was later frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The lyophilized extract (10 mg) was redissolved in 2 mL of ethanol/water (20:80, v/v) to obtain a final concentration of 5 mg/mL.

The phenolic profile was determined by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). These compounds were separated and identified as previously described by Bessada et al. (2016). The obtained extracts were re-dissolved at a concentration of 10 mg/mL with the methanol: water (80:20, *v/v*) mixture. A double online detection was performed using a DAD (280, 330 and 370 nm as preferred wavelengths) and a mass spectrometer (MS). The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source.

The identification of the phenolic compounds was performed based on their chromatographic behaviour and UV-vis and mass spectra by comparison with standard compounds, when available, and data reported in the literature giving a tentative identification. Data acquisition was carried out with Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA). For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV-vis signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard: caffeic acid ($y = 388345x + 406369$, $R^2 = 0.9939$, limit of detection (LOD) = 0.78 µg/mL, limit of quantification (LOQ) = 1.97 µg/mL),

catechin (y = 84950x – 23200; R^2 = 1; LOD = 0.17 µg/mL; LOQ = 0.65 µg/mL), chlorogenic acid (y = 168823x – 161172, R^2= 0.9999; LOD = 0.20 µg/mL; LOQ = 0.68 µg/mL); and isoliquiritigenin (y=42820x + 184902, R^2 = 0.999, LOD = 0.018 µg/mL; LOQ = 0.054 µg/mL). The results were expressed as mg/g of extract.

2.5 Evaluation of bioactive properties

2.5.1 ***Preparation of the hydroethanolic extract***

The extraction was performed as described above in section 3.4.4. The lyophilized extract was redissolved at a concentration of 10 mg/mL in ethanol/water (80:20, v/v) for the antioxidant activity assays (section 3.5.2.), in culture medium (10 mg/mL) for the analysis of antimicrobial activity (section 3.5.3.) and in distilled water at a concentration of 8 mg/mL for the evaluation of cytotoxic activity (section 3.5.4.) and hepatotoxicity (section 3.5.5.). Subsequently, these solutions were successively diluted in order to obtain working concentrations.

2.5.2 ***Antioxidant activity***

DPPH free radical scavenging activity: In a 96-well microplate (**Figure 10**), 30 µL of hydroethanolic extract with different concentrations, to which a methanolic solution of DPPH (6 x 10^{-5} mol/L; 270 µL) was added. Then, the plate was placed in the dark for 60 minutes for the absorbance values to stabilize, since the reduction of the DPPH radical is evaluated by measuring the absorbance at 515 nm, in a microplate reader (Bio-Tek ELX800; Bio-Tek Instruments Inc., Vermont, USA).

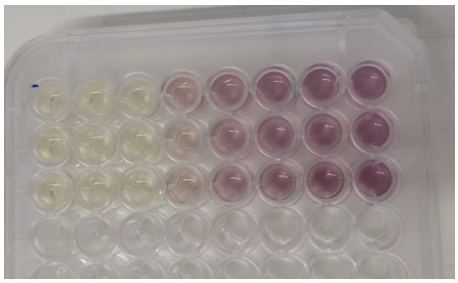

Figure 10. Determination of free radical scavenging activity (Author authorship).

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Free radical scavenging activity (RSA) was calculated using **Equation 2**:

$$\% \text{ RSA} = [(A_{DPPH} - A_S) / A_{DPPH}] \times 100$$

Equation 2. Equation for determining RSA in the DPPH method.

Where:

A_S is the absorbance of the solution in the presence of extract at a given concentration

A_{DPPH} is the absorbance of the DPPH solution.

The extract concentration corresponding to 50% of radical scavenging activity (EC_{50}) was calculated from the graphic representation of the percentage of RSA as a function of extract concentration. The control used in this assay was trolox (Barros et al., 2010).

Reducing power activity: This methodology was performed using the previously described microplate reader. Extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L; pH 6.6; 0.5 mL) and potassium ferricyanide (1 % m/v; 0.5 mL). The mixture was incubated at 50°C for 20 min, then trichloroacetic acid (10% w/v; 0.5 ml) was added. The mixture (0.8 mL) was placed in a 48-well microplate (**Figure 11**), as well as deionized water (0.8 mL) and iron chloride (0.1% w/v; 0.16 mL), and then the absorbance at 690 nm was measured.

The extract concentration that provides 0.5 absorbance (EC_{50} - 50% of the maximum absorbance value in which the Lambert-Beer Law is applied) was calculated from the graphical representation of the absorbance at 690 nm as a function of the extract concentration. In this assay, trolox was used as a control (Barros et al., 2010).

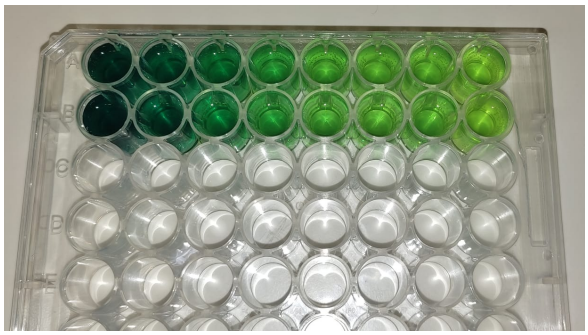

Figure 11. Microplate used for the evaluation of reducing power.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS), to which tris-HCl buffer was added (20 mM, pH=7.4), homogenized and centrifuged by 10 min (3500 rpm). Extracts of different concentrations (10, 5, 2.5, 1.25, 0.625, 0.325mg/mL) contained in the test tubes (200 µL) were added along with 100 µL of ascorbic acid, 100 µL of iron sulfate and 100 µL of brain suspension supernatant. The tubes were placed in a bath at 37 °C for 1 hour, at the end of which 500 µL of trichloroacetic acid (28%) and 380 µL thiobarbituric acid (2%). The tubes were then incubated at 80 °C for 20 minutes. After centrifugation at 3000rpm for 5 min for protein removal, the colour intensity of the malonaldehyde complex (MDA) -TBA of the supernatant was measured by absorbing it at 532 nm (**Figure 12**).

The inhibition ratio (%) was calculated using the **Equation 3**:

$$Inhibition\ ratio\ (\%) = [(A - B)/A] \times 100$$

Equation 3. Equation for determining the inhibition ratio.

Where,

A: the absorbance of the control

B: the compound solution

The results were presented in IC_{50} values that represent the sample concentration that provides 50% of antioxidant activity (Barros et al., 2010).

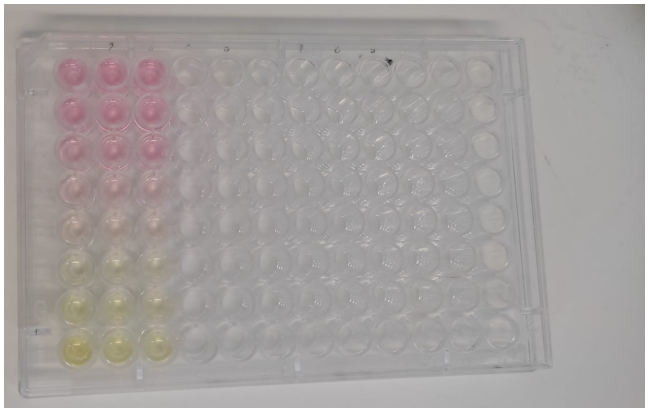

Figure 12. Microplate used for the evaluation of TBARS (Author authorship).

Oxidative haemolysis inhibition assay (OxHLIA)

The antihaemolytic activity of the extracts was evaluated by the oxidative haemolysis inhibition assay (OxHLIA) described previously by Takebayashi et al. (2012) with some modifications by Lockowandt et al. (2019). Sheep blood samples were collected from healthy animals and centrifuged at 1000 g for 5 min at 10 °C. Plasma and buffy coats were discarded and erythrocytes were first washed once with NaCl (150 mM) and three times with phosphate-buffered saline (PBS, pH 7.4) (Evans et al., 2013). The erythrocyte pellet was then resuspended in PBS at 2.8% (v/v). Using a flat bottom 48-well microplate, 200 µL of erythrocyte solution was mixed with 400 µL of either PBS solution (control), antioxidant sample dissolved in PBS, or water (for complete haemolysis). Trolox was used as positive control (7.81–250 µg/mL PBS). After pre-incubation at 37 °C for 10 min with shaking, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 160 mM in PBS, 200 µL) was added and the optical density was measured at 690 nm.

After that, the microplate was incubated under the same conditions and the optical density was measured every 10 min at the same wavelength for approximately 400 min (Takebayashi et al., 2012). The percentage of the erythrocyte population that remained intact (P) was calculated according to **Equation 4**:

$$P\ (\%) = (S_t - CH_0 / S_0 - CH_0) \times 100$$

Equation 4. Percentage of the erythrocyte (PE) population in the OxHLIA assay.

Where S_t and S_0 correspond to the optical density of the sample at t and 0 min, respectively, and CH_0 is the optical density of the complete haemolysis at 0 min. The results were expressed as delayed time of haemolysis (Δt), which was calculated according to **Equation 5**:

$$\Delta t \text{ (min)} = Ht_{50} \text{ (sample)} - Ht_{50} \text{ (control)}$$

Equation 5. Haemolysis delay time.

Where Ht_{50} is the 50% hemolytic time (min) graphically obtained from the haemolysis curve of each antioxidant sample concentration. The Δt values were then correlated to antioxidant sample concentrations (Takebayashi et al., 2012) and, from the correlation obtained, the extract concentration able to promote a Δt haemolysis delay was calculated. The results were given as IC_{50} values (μg/mL) at Δt 60 and 120 min, i.e., extract concentration required to keep 50% of the erythrocyte population intact for 60 and 120 min.

*2.5.3 **Evaluation of antimicrobial activity***

Antibacterial activity

Food contaminats (bacteria): The extracts were tested against five Gram-negative bacteria, namely, *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 25923). All these microorganisms are purchase at Frilabo, Porto, Portugal. The bacterias were incubated at 37 ºC an appropriate fresh medium, for 24 h before analysis to maintain the exponential growth phase.

Clinical bacteria: The bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Morganella morganii and* three Gram-positive bacteria (*Enterococcus faecalis, Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus (MRSA*), were tested.

The MIC determinations on all bacteria were conducted using colorimetric assay according to described by Pires et al, 2018. The samples were first of all dissolved in 5% (v/v) Dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20 mg/ mL for the stock solution. 90 µL of this concentration was added in the first well (96-well microplate) in duplicate with 100 µL of Tryptic Soy Broth (TSB). In the remaining wells 90 µL of TSB medium were added. Then the samples were serially diluted to obtain the concentration ranges (10 to 0.03125 mg/mL). To finish, 10 µL of inoculum (standardized at 1.5×10^6 Colony Forming Unit (CFU) /mL) was added at all the well assuring the presence of 1.5×10^5 CFU. Two negative controls were prepared, one with TSB and another one with the extract. Two positive controls were prepared with TSB and each inoculum and another with medium, antibiotics, and bacteria. Ampicillin and Steptomicin were used for all bacteria tested and Meticilin was also used for *Staphylococcus aureus.* The microplates were incubated at 37°C for 24 h. The MIC of samples was detected following addition (40 µl) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37°C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determinate by change the coloration from yellow to pink if the microorganisms are viable. For the determination of MBC, 10 µL of liquid from each well that showed no change in colour was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37°C for 24 h. The lowest concentration that yielded no growth determines the MBC. MBC was defined as the lowest concentration required to kill bacteria.

Antifungal activity

The antifungal activity was performed according to described by Heleno et al., 2013. *Aspergillus fumigatus* (ATCC 204305), *Aspergillus brasiliensis* (ATCC 16404) were used. The organisms were obtained from Frilabo, Porto, Portugal. The micromycetes were maintained on malt agar and the cultures stored at 4 °C and were further placed in new medium and incubated at 25ºC for 72h. In order to investigate the antifungal activity, the fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well. The samples were first of all dissolved in 5% (v/v) Dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20 mg/ mL for the stock solution. Afterwards, 90 µL of this concentration was

added in the first well (96-well microplate) in duplicate with 100 µL of Malt Extract Broth (MEB).

In the remaining wells 90 µL of medium MEB were placed. Then the samples were serially diluted obtain the concentration ranges (10 to 0.03125 mg/mL). Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microplate. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentration (MFC) was determined by serial subcultivation of a 2 µL of tested compounds dissolved in medium and inoculated for 72 h, into microplates containing 100 µL of MEB per well and further incubation 72 h at 26 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicide ketoconazole (Frilabo, Porto, Portugal), was used as positive control.

2.5.4 ***Evaluation of toxicity and antiproliferative activity***

The extract effects on the growth of human tumor cell lines were evaluated by sulforhodamine B (SRB) assay in order to determine cell growth inhibition. For this, 4 tumor cell lines were used: MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), AGS (gastric adenocarcinoma), CaCo (colorectal adenocarcinoma).

The cells were maintained as adherent cultures in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (MCF-7, NCI-H460, AGS and CaCo-2) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin, at 37 °C in an incubator with humidified air and 5% CO_2. Each cell line was prepared at the appropriate density (1.0×10^4 cells/well) in 96 well plates and left to adhere for 24 h. After this time the cells were tested for 48h with various concentrations of extracts. After the incubation period, the adherent cells were fixed by adding 10% cold trichloroacetic acid (TCA, 100 µL) and left to stand for 60 min at 4 ºC. The plates were then washed with deionized and dried water.

To each well 100 µL of the SRB solution (0.1% in 1% of acetic acid) were added and the plates were allowed to incubate at room temperature for 30 min. Excess RBS was removed by washing the plates with 1% acetic acid and were left to air dry. Then the SRB on was solubilized with 10 mM Tris (200 µL) and the absorbance was measured at 540 nm in a microplate reader (Biotek Elx800). The dose-response curves were obtained for each extract and cell line tested. The GI_{50} value was also calculated, corresponding to the concentration of extract that inhibits

50% of cell growth (Vichai & Kirtikara, 2006; Abreu et al., 2011). Ellipticine was used as a positive control.

For non-tumor cells, a cell culture was prepared from the kidney of an African green monkey, which was called VERO. The kidney tissue was washed with Hank's balanced salt solution containing 100 U/mL of penicillin, 100 µg/mL of streptomycin and divided into 3 explants of 1×1 mm. Some of these explants were placed in tissue vials of 25 cm^2, with DMEM medium supplemented with 10% bovine fetal serum, 2 mM of non-essential amino acids and 100 U/mL of penicillin, 100 mg/mL of streptomycin and incubated at 37 ºC with humidified atmosphere and 5% CO_2. The medium was changed every 2 days. Cell culture was continued with direct monitoring every 2 or 3 days using a phase contrast microscope. Subsequently, a cell subculture was performed, and these were placed in plates of 96 wells with the density of 1.0 x10^4 cells/well and cultivated in DMEM medium with FBS 10%, 100 U/mL of penicillin and 100 mg/mL of streptomycin (Abreu et al., 2011).

2.6 Incorporation of apple flour as natural ingredients in bread

To evaluate the potential of apple flour, as a potential natural ingredient, it was incorporated into two types of bread (wheat bread and corn bread) usually prepared at the Pão de Gimonde bakery in Bragança (Portugal). The effects of this incorporation on different parameters throughout its shelf life were evaluated.

2.6.1 ***Preparation of bread***

For wheat bread: 10 g of yeast were dissolved in 600 g of water and 15 g of salt were added and mixed with 900 g of wheat flour until a homogeneous dough was obtained. The dough was covered with cling film and left to rise for 30 minutes in a warm place. After molding the bread shape, it is taken to the preheated oven for 30 minutes at 200ºC.

For wheat bread with apple flour the above process was followed by adding 100 grams of apple flour to the wheat flour. For corn bread: 10 g of yeast were dissolved in 600 g of water and 15 g of salt were added and mixed with 900 g of corn flour until a homogeneous dough was obtained. The dough was covered with cling film and left to rise for 30 minutes in a warm place. After molding the bread shape, it is taken to the preheated oven for 30 minutes at 200ºC. For

the apple flour corn bread, the above process was followed by adding 100 grams of apple flour to the corn flour.

All samples (**Table 2**) were lyophilized, finely crushed and analysed, in triplicate, immediately after preparation (T0) and after three (T3) and five (T5) days of storage (at room temperature and packed in a sealed plastic bags covered with aluminum paper).

Table 2. Different samples of bread prepared (Author authorship)..

	Traditional wheat bread	Wheat bread with apple flour	Traditional corn bread	Corn bread with apple flour
External appearance				
Interior appearance				

Physical parameters

For each bread sample the water activity "a_w" was determined at 20 °C for 5 min at the surface of each sample slice by using an activity meter instrument (AQUALAB 4TE) based on the dew-point method and with an absolute error of 0.003. The average a_w value of each slice was calculated from the a_w values estimated at its surface and at the adjacent slice surface (**Figure 13**).

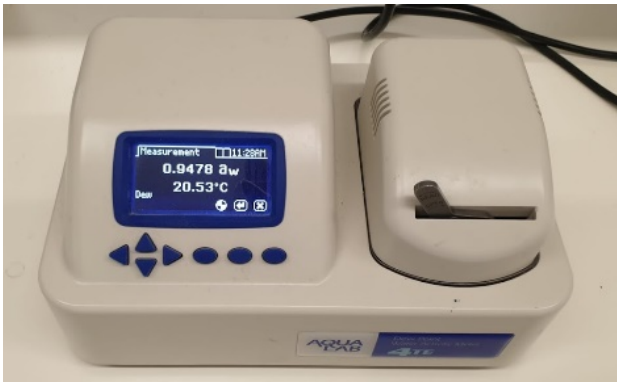

Figure 13. Water activity determination (Author authorship).

The colour of the samples was measured in triplicate by each group of samples, at three different points using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The Illuminate C was used and an 8 mm diaphragm aperture was previously calibrated against a standard white tile. CIE *L** (luminosity), *a** (green/red), *b** (blue/yellow) colour values were recorded using the data software "Spectra Magic Nx" (Fernandes et al., 2012).

Texture evaluation was performed using a TA.XT Plus texture analyzer (**Figure 14**) with a 30 kg Stable Micro Systems load cell (Vienna Court, Godalming, UK) via a P/45 aluminum cylinder, which performed a human mouth-like lateral analysis (TPA) by pressing twice on the matrix. At pre- and post-test velocities set at 3 mm/sec and the target position was set to 25% pressure starting at 50 g of force. Expressing results by macro has been combined to reach different dimensions of texture, namely, hardness, adhesion, quaternity, cohesion, chewiness, and elasticity. The results were analysed by Exponent software (Carocho, et al., 2020).

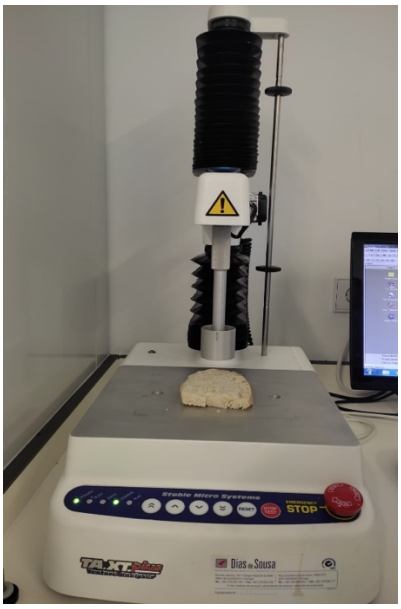

Figure 14. The TA.XT Plus texture analyzer(Author authorship).

2.6.2 **Determination of the proximate composition of bread**

The nutritional composition (protein, fat, carbohydrates, ash, humidity, and the total energy value) of bread samples was determined according to official food analysis methodologies (AOAC, 2016).

The water content of each loaf was measured using a PMB moisture analyzer (Kingston, Milton Keynes, UK) (**Figure 15**). 2 g of sample was used and each measurement was performed in triplicate.

Figure 15. PMB moisture analyzer (Author authorship).

To determine the content of protein, fat, ash, carbohydrates and the total energy value, the procedures previously described in section 3.3 were followed.

2.6.3 **Determination of the sugars and fatty acids of the bread**

For the determination of free sugars and fatty acids of different bread samples, the methodologies previously described in sections 3.4.1 and 3.4.3., respectively, were followed.

2.6.4 **Microorganism Analysis**

The preparation of samples for the microbial load analysis followed the procedure described in the International Organization for Standardization (ISO, 2003). The different nutraceutical preparations (5 mL) were mixed with 45 mL of peptone water in stomacher bags and further homogenized in a stomacher equipment (Star Blender, VWR, Radnor, USA) for 1 min at 300 units. The obtained suspensions were further diluted to obtain dilutions from 10-1 to 10-5. Each solution was analyzed in duplicate.

Total aerobic mesophiles: the incorporation sowing technique was used: 1 mL of each dilution the sample was placed in a Petri dish and 15 mL of Plate Count Agar (PCA), were added. The procedure was performed in duplicate. The plates were then homogenized and after solidification of the medium, they were incubated at 30 °C in an inverted position for 72 h. Counting was performed on plates containing between 15 and 300 colonies (Limit of Quantification (LOQ) = 1 log (Colony Forming Units) CFU/g).

Coliforms (and E. coli; ISO 4832:2006): The dilutions were inoculated in VRBLA (violet red bile lactose agar) (Liofilchem Co., Roseto degli Abruzzi, Italy) by the pour plate technique, in duplicate (Limit of Quantification (LOQ) = 1 log (Conoly-Forming Units) CFU/g): 1 mL of suspension was pipetted into the plate and 15 mL of melted VRBLA (Liofilchem Co., Roseto degli Abruzzi, Italy) (kept at 50 °C in a water bath or incubator) were poured prior to homogenization, and left to solidify. On the top of the medium, a layer of 4 mL of VRBLA was poured and was left to solidify. The plates were incubated at 30 °C for 48 h, in reversed position. Counting was performed in plates that had between 10 and 150 colonies.

Yeasts and Moulds (ISO 21527-1/2:2008): The dilutions were inoculated in DRBC (dichloran rose bengal chloramphenicol) (Liofilchem Co., Roseto degli Abruzzi, Italy) by the spread plate technique, in duplicate (LOQ = 1.7 log CFU/g): 0.2 mL of the suspensions were pipetted onto a plate containing 15 mL of the medium and were spread with a disposable spreader. Incubation was set at 25 °C for 5 days, in the upright position. In the plates having less than 150 colonies, the count of yeast and mold colonies was performed separately after 2 and 5 days of incubation.

Bacillus cereus (ISO 7932:2004): The dilutions were inoculated in MYP (mannitol yolk polymyxin) (Liofilchem Co., Roseto degli Abruzzi, Italy) by the spread plate technique, in duplicate (LOQ = 1.7 log CFU/g): 0.2 mL of suspension were pipetted onto a plate containing 15 mL of the medium and were spread with a disposable spreader. Incubation was performed at 30 °C for 24–48 h, in reversed position. Counting was performed in the plates having between 10 and 150 colonies. All counts of the microbial load were performed at the same intervals as all other assays of this work, namely T0—immediately after preparation and contamination, T30—after 30 days which was the shelf-life of the nutraceutical after opening, and T45, which was beyond the shelf-life.

2.6.5 **Statistical analysis**

Throughout the whole document, all data was expressed as mean ± standard deviation. Furthermore, to better understand the effect of the addition of the apple flour storage time, for all analyses the samples were analyzed through a two-way ANOVA with type III sums of squares using the SPSS Software, version 25. This multivariate general linear model treats the two factors, incorporation (I) and storage time (ST) as independent, thus allowing the effect of each one to be analyzed independently, providing more insight on their contribution towards the outcome. If a significant interaction (<0.05) was recorded among the two factors (I×ST), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM), when possible. If there was no significant interaction (>0.05), each factor was evaluated independently using a simple Student's T test (for I) or a Tukey's multiple comparison test (ST) when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

3 Results and Discussion

4.1. Apple Flour

As the main objective of this study is the technological enhancement of apple by-products through the development of a new product, in this particular case, apple flour, it is extremely important to carry out a chemical and nutritional assessment. Like the apple, it is expected that the application of this new product will also bring nutritional benefits to the consumer.

The results of the nutritional composition of apple flour obtained from Fuji apple residues are shown in **Table 3**.

Table 3. Nutritional composition of apple flour (mean ± SD).

Moisture (%)	6.5±0.1
Fat (g/100g)	0.061±0.003
Ash (g/100g)	1.5±0.1
Proteins (g/100g)	3.959±0.002
Carbohydrates (g/100g)	97.5±0.1
Energy (Kcal/100g)	394.5±0.2

After drying, a large reduction in moisture content was achieved. The product that initially had a moisture content of 84.2% now has a moisture content of 6.5% and therefore much of the water has been removed from the by-products. Thus, through the drying process, 77.7% of the water contained in the by-product was removed. According to the food composition table of the National Institute of Health Dr. Ricardo Jorge, wheat flour (type 55) (flour traditionally used by the bakery and pastry industry) has a moisture content of 13.40%. Therefore, the apple flour obtained in this study has lower moisture content than wheat flour (type 55). In this way, it is considered that the drying process was efficient, since the prepared apple flour presented a lower moisture content value than the wheat flour (type 55) which is an essential parameter also in the conservation of the product.

Table 3 reveals that apple flour has a fat content of 0.061±0.003 g/100g. A study by Serra et al. (2021) reported a higher value in Fuji apple flour with a value of 0.54 g/100g). In

turn, a study developed by Coelho and Wosiacki (2010) showed a lipid value of 1.31 g/100g in apple pomace flour.

Considering **Table 3**, it was found that the ash content in apple flour is 1.5±0.1 g/100g. According to the food composition table of the National Institute of Health Dr. Ricardo Jorge, 100 g of dried apple contains approximately 1.4 g of ash, and therefore, the result obtained is relatively close to what is mentioned in the literature. This value agrees with a study carried out previously by Coelho and Wosiacki (2010) in which he described an ash content of 1.4% in apple pomace. The same ash value in apple flour was recorded by Zlatanovic et al (2019). In turn, Serra et al. (2021) in a comparative study of 5 varieties of apple pomace flour described ash values ranging between 1.2 and 1.9 (g/100g). According to the National Institute of Health food composition table Dr. Ricardo Jorge, wheat flour (type 55) contains an ash content of 0.50 g per 100 g of product. From this analysis it can be concluded that apple flour has a higher content than wheat flour.

Regarding the protein content (**Table 3**), the apple flour analysed in this study presented 3.959±0.002 g/100g of protein. These values appear in agreement with some studies found in the literature that nutritionally evaluate apple pomace flours. Zlatanovic et al (2019) present a protein content that varies between 3.2 and 5.8 g/100g in apple pomace flours. Similarly, Coelho and Wosiacki (2010) described a protein content of 3.35 g/100g in apple pomace flour. In the study developed by Sato (2010), apple pomace from eleven cultivars were compared and the protein content values ranged from 3.75 to 4.65 g 100/g,

Fructose, glucose and sucrose were the free sugars found in the apple flour obtained in this study (**Table 4**). According to the values obtained, the sugar with the highest content in the flour was fructose in an amount of 17±1 g/100g.

Table 4. Sugars of apple flour (mean ± SD).

Free sugar	Amount (g/100g)
Frutose	8.9±0.5
Glucose	6.9±0.4
Sucrose	1.18±0.04
Total	17±1

The sugar content in apples can vary depending on different conditions, which may explain the differences found in the different studies presented in the literature. Apples exposed to sunlight contain a higher percentage of sugar compared to fruits in the shade. Likewise, sugar content can also be affected by harvest periods (Nilsson and Gustavsson, 2007). In the study developed by Coelho and Wosiacki (2010), apple pomace flour had higher total sugar content (35.11 g/100g), with 22.31 g/100g of glucose being highlighted. When comparing apple pomace flours, Zlatanovic et al (2019) showed glucose values that ranged between 9 and 18 (g/100g), fructose between 21 and 35 (g/100g) and sucrose between 5.6 and 9.7 (g/100g). /100g).

Table 5 presents the composition of organic acids in apple flour. In this study, six organic acids (oxalic, quinic, malic, citric and fumaric acids) were identified in apple flour.

Table 5. Organic acids of apple flour (mean ± SD).

Organic Acid	Amount (mg/g)
Oxalic acid	2.7±0.2
Quinic acid	5.1±0.7
Malic acid	20±1
Citric acid	4±1
Fumaric acid	0.10±0.01
Total	32±3

Malic acid emerged as the major organic acid in apple flour. This organic acid emerged predominating along with citric acid in Fuji apples (Wu et al., 2007). In a comparative study between different apple species, malic acid content ranged between 1.72 and 2729 mg/g (Ma et al., 2018). Likewise, Pires, et al (2018) highlighted the majority presence of malic acid followed by quinic and oxalic acids (1.36, 0.15, 0.101 mg/100 g, respectively) in “Bravo de Esmolfe” apples.

The individual composition of fatty acids as well as the content of saturated, monounsaturated and polyunsaturated fatty acids is shown in **Table 6**. The results show an outstanding amount of unsaturated fatty acids, due to the high contribution of oleic acid.

Table 6. Fatty acids of apple flour (mean ± SD).

Compound name	Amount (%)
C10:0	0,35±0,01
C11:0	0,64±0,03
C12:0	0,51±0,03
C15:0	0,32±0,02
C15:1	0,95±0,04
C16:0	19±1
C16:1	0,77±0,02
C17:0	0,45±0,02
C18:0	5,3±0,2
C18:1n9	38,8±0,1
C18:2n6	27±1
C18:3n3	1,64±0,03
C20:1	3,95±0,08
C22:0	0,63±0,03
SFA	27,3±0,7
MUFA	44,47±0,06
PUFA	28,2±0,7

Results are presented as mean ± standard deviation. Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecylic acid (C11:0); Lauric Acid (C12:0); Pentadecylic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Margaric Acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); Linolenic acid (C18:3n3); Eicosenoic acid (C20:1); Behenic Acid (C22:0); SFA- Saturated Fatty Acids; MUFA- Monounsaturated Fatty Acids; PUFA- Polyunsaturated fatty acids.

The apple flour had fourteen fatty acids in its composition, highlighting the oleic (C18:1n9), linoleic (C18:2n6) and palmitic (C16:0) acids as the major fatty acids in a percentage of 38.8 ± 0.1%, 27 ± 1% and 19 ± 1%, respectively. This predominance is responsible for the prevalence of unsaturated fatty acids in relation to saturated fatty acids in this flour. These results are in agreement with the data presented in a comparative study of eight apple cultivars (Wu et al., 2007) where linoleic acid appears as the majority. In a study developed by Pires, et al. (2018) analysing the acid composition of "Bravo de Esmolfe" apples, palmitic and linoleic acid predominated with 28.94% and 15.8%, respectively.

The chromatographic responses (retention time, wavelengths of maximum absorption, and mass spectral data) and tentative identification of the phenolic compounds present in the apple flour extract are described in **Table 7**. The corresponding quantification is also present in **Table 7**. Seven phenolic compounds were found, divided in three different families of compounds: three phenolic acids (caffeic acid and caffeoylquinic acid derivatives), two flavan-3-ols ((epi) catechin derivatives), and two dihydrochalcone (phloretin *O*-glycosides).

The phenolic profile of apple fruits has been extensively studied by other authors (Arraibi et al., 2021; Cao et al., 2009; De Paepe et al., 2013; Montero et al., 2013; Pires et al., 2018; Sánchez-Rabaneda et al., 2004; Wojdyło et al., 2008) However, as far as the authors knowledge, this is the first study of the phenolic profile of apple flour. Overall, the profile found is coherent with the previous published ones, apart from the fact that the number of phenolic compounds is very low. This could be due to processing steps regarding the flour preparation that can lead to the degradation of the compounds.

Regarding their amounts, peak **1**, tentatively identified as caffeic acid hexoside, was the major peak found, with 0.71±0.01 mg/g extract. Consequently, the total phenolic acids group presented the major concentration, 1.217±0.001 mg/g extract, which is in coherence with the results presented by Arraibi et al. (2021) in hydroethanolic and aqueous extracts of Spanish and Belgian apple pomace. The flavan-3-ols group was the second major group found, also in coherence with the results presented by Arraibi et al. (2021), however, at very low concentrations. Finally, the dihydrochalcone group, represented by phloretin *O*-glycosides, despite present, were found in trace amount in the studied sample. Again, this can be due to the processing steps needed for flour preparation. Nonetheless, the presence of this type of compounds is always very important, due to the pharmacological properties of this compounds (Stompor, Broda, & Bajek-Bil, 2019), that can add value to matrix sample in which they are found.

The amount of total phenolic content present in apple flour was 1.4119±0.0004 mg/g extract, being the most representative compounds are phenolic acids. Ćetković et al. (2008) in a comparative study of different apple pomace shows a total phenolic content that varies from 0.692 and 1.474 (mg/g). Also, Feng et al. (2021) showed in their study in apple pulp from *Malus domestica* Borkh a value of total phenolic compounds ranging between 0.096 and 0.416 (mg/g). Although the study by Pires et al. (2018) highlighted the presence of 15 phenolic compounds in 'Bravo de Esmolfe' apples, which is significantly higher than the composition of our apple flour, 5-O-caffeoylquinic acid was also the major compound (51.5 mg/100 g dw).

Table 7. Retention time (Tr), wavelengths of maximum absorption in the UV-Vis region (ʎmax), attempt to identify and quantify phenolic compounds in apple flour extract (mean ± SD).

Peak	Rt (min)	lmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	mg/g extract
1	4,87	364	341	179(100),161(34),135(5)	Caffeic acid hexoside	0.71±0.01
2	6,66	322	353	191(12),179(1),173(100),161(1),135(2)	4-*O*-caffeoylquinic acid	0.29±0.01
3	7.24	327	353	191(100),179(6),173(2),161(1),135(1)	5-*O*-caffeoylquinic acid	0.212±0.004
4	8,58	281	577	451(24),425(100),407(21),289(12)	B-type (epi)catechin dimer	0.118±0.003
5	11.39	280	865	739(74),713(44),695(100),577(64),575(37),425(10),407(9),289(8),287(7)	B-type (epi)catechin trimer	0.077±0.002
6	18,08	285	567	273(100)	Phloretin-2-*O*-xyloglucoside	tr
7	22.89	285	435	273(100)	Phlorizin (phloretin-2-*O*-glucoside)	tr
					TPA	1.217±0.001
					TF3O	0.195±0.001
					TdhC	tr
					TPC	1.4119±0.0004

TFA - Total phenolic acids; TF3O – Total Flavan-3-ol; TdhC - Total dihydrochalcone; TPC - total phenolic compounds; tr - traces. Calibration curves: caffeic acid (y = 388345x + 406369, R^2 = 0.9939, peak 1), chlorogenic acid (y = 168823x - 161172, R^2 = 0.9945, peak 2 and 3), catechin (y = 84950x - 23200, R^2 = 0.9968, peak 4 and 5) and isoliquiritigenin (y = 42820x + 184902, R^2 = 0.9964, peak 6 and 7).

The antioxidant activity was evaluated through four in vitro assays: DPPH radical scavenging activity, reducing power, inhibition of the formation of thiobarbituric acid reactive substances (TBARS) and inhibition of oxidative hemolysis (OxHLIA). The results are shown in **Table 8** and displayed as EC_{50} values (value representing the sample concentration that provides 50% antioxidant activity). Considering that the lower the EC_{50} value, the greater the antioxidant activity (Arbos, Freitas, Stertz and Dornas, 2010), it appears that apple flour showed greater antioxidant activity than trolox in the reducing power test and similar values for the DPPH and TBARS assays. The presence of phenolic compounds in numerous vegetables, fruits, and natural plants is thought to be responsible for their antioxidant properties. Polyphenols' radical-scavenging capacity is determined by their molecular structure and the availability of phenolic hydrogens (Birasuren et al 2013).

In the literature, several studies have reported the excellent antioxidant activity of several species of apples and by-products. Hamauzu et al. (2005) and Luo et al. (2016) have reported lower EC_{50} values when using the DPPH scavenging activity methodology in Malus domestica (EC_{50}= 8.4 mg/100 ml and 0.26 mg/ml, respectively).

Table 8. Antioxidant activity of the hydroethanolic extract of apple flour (mean ± SD).

	DPPH scavenging activity (mg/mL)	**Reducing power (mg/mL)**	**TBARS (mg/mL)**	**OxHLIA(mg/mL)**	
				Δt 60 min	Δt 120 min
Apple flour	4,7±0,2	2,44±0,04	0.64±0.02	232±8	566±10
Trolox	4.2±0.1	4.1±0.1	0.54±0.8	21.8±0.3	44±1

The antibacterial activity of hydroethanolic extracts obtained from apple flour was tested against a panel of eight food bacteria (*Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterocolitica*, *Yersinia enterocolitica*, *Bacillus cereus*, *Listeria monocytogenes* e *Staphylococcus aureus*) and oito clinical bacterial strains (*Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Listeria monocytogenes* e *MRSA*). **Table 9** presents the results obtained for each extract in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The food bacteria most sensitive to apple flour were *Yersinia enterocolitica* and *Staphylococcus aureus* with lower MIC values (0.6 mg/mL). In turn, the most sensitive clinical bacteria were *Escherichia coli* and MRSA with MICs of 1.25

mg/mL. However, apple flour was not bactericidal for any of the bacteria tested up to the maximum concentration tested (10 mg/mL).

Table 9. Antibacterial and antifungal activity of apple flour extract (MIC, MBC and MFC in mg/mL).

			Positive Control					
	Apple flour		Streptomicin 1mg/mL		Methicilin 1mg/mL		Ampicillin 20mg/mL	
ANTIBACTERIAL ACTIVITY	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Food Bacteria								
Gram-negative bacteria								
Enterobacter Cloacae	>10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterocolitica	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	0.6	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria								
Bacillus cereus	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	0.6	>10	0.007	0.007	0.007	0.007	0.15	0.15
Clinical bacteria								
					Positive control			
	Apple Flour		Ampicillin (20mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria								
Escherichia coli	1.25	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	>10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	2.5	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria								
Enterococcus faecalis	2.5	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	1.25	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

			Ketaconazole 1mg/mL	
ANTIFUNGAL ACTIVITY	MIC	MFC	MIC	MFC
Aspergillus brasiliensis	10	10	0.06	0.125
Aspergillus fumigatus	10	10	0.5	1

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MFC: Minimum fungicidal concentration.

The antifungal activity of the hydroethanolic extracts obtained from apple flour was tested against a panel of two fungi (*Aspergillus brasiliensis* e *Aspergillus fumigatus),* and the results expressed in minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC), being presented in the **Table 9.** The apple flour showed the ability to inhibit the growth of both tested fungi (MIC and MFC of 10 mg/mL).

There are several studies that have reported promising antimicrobial activity of different apple varieties and by-products, namely apple pomace. Luo et al. (2016) and Pires et al. (2018) highlight the excellent antimicrobial activity of apples of the Fuji and "Bravo de Esmolfe" varieties, respectively. Fuji apples had lower MIC values than "Bravo de Esmolfe" and our apple flour, since the strains used in the present study and in the study by Pires et al. (2018) were obtained from clinical isolates with multi-resistant profiles.

The activity of apple flour on the growth of the four human tumor cell lines (MCF-7, NCI-H460, AGS and CaCo) and for the primary culture of non-tumor cells (VERO) is presented in **Table 10** and are expressed in values of the concentration of extract responsible for inhibiting cell proliferation by 50% (GI_{50}), values in μg/mL. The results showed that apple flour only showed positive results (GI_{50} <400 μg/mL) demonstrating the ability to inhibit the growth of the NCI-H460 (lung cancer) cell line. The results also allow to verify that the apple flour extract did not express cytotoxicity against non-tumor cell culture, VERO (GI_{50} > 400 μg/mL).

Schaefer et al. (2006) reported a decrease in DNA damage of CaCo2 cells when treated with apple juice, which led to the conclusion that apple consumption, may serve to protect against colon cancer risk by protecting colonic cells against DNA damage and abnormal extracellular behaviour.

Table 10. Results of toxicity and antiproliferative activity of apple flour (mean ± SD).

		Apple flour	**Ellipticine (μM)**
Tumoral cell lines	AGS	>400	1.23±0.03
	CaCo-2	>400	1.21±0.02
	MCF_7	>400	1.02 ± 0.02
	NCI-H460	53±5	1.01±0.01
Non-tumoral culture	VERO	>400	0.6±0.1

GI_{50} values correspond to the concentration that causes 50% inhibition of cell proliferation; AGS - human gastric adenocarcinoma; CaCo-2 - human colon adenocarcinoma MCF-7 - human breast adenocarcinoma; NCI-H460 - human lung carcinoma.

4.2. Alternative breads

Considering the results and discussion, the following tables are presented with two levels, the top referring to the case of the corn bread and the bottom to the wheat bread. As detailed in the statistics section of the materials and methods, the tables are represented in such a way that the values of the incorporation (I) are represented as means of each storage time (ST) including both incorporation 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 it includes the variation of the non-fixed factor (I or ST). If a significant interaction among these two factors is detected (I×ST $p<0.05$), no multiple comparisons can be carried out, meaning that both factors, I and ST contributed to the changes in the breads, hindering concrete conclusions, although general tendencies can sometimes 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, for ST, and a Student's T test for I.

Table 11 represents the three colour coordinates (L^*, a^* and b^*) for the different sections the breads, namely the top and bottom section, and also the crumb. Among both breads, there was a significant interaction among the breads in for all colour coordinates, which only allows general conclusions to be extracted from the EMM.

Figure 16 represents the colour coordinates of the top of the corn bread, in which the control bread showed a higher lightness (L^*), but lower redness (a^*) and higher blueness (b^*), in the top section, while showing a lighter hue also for the bottom section. Regarding the crumb of the bread made with corn, the apple incorporation seemed to increase the redness and blueness (**Figure 17**). Regarding the bread made with wheat, similar results were sought in terms of the colours of the top and the bottom (**Figure 18**). The crumb of the wheat bread fortified with apple showed lower differences to the control, especially in terms of the yellow blueness. Overall, there were some interesting changes in terms of the bread colours meaning the addition of the apple flour could increase the intensity of the bread colour, which is always an appealing asset for innovation in the baking industry.

Table 11. Colour coordinates (*L**, *a**, and *b**) of the different sections of both breads.

		L* Top	a* Top	b* Top	L* Bottom	a* Bottom	b* Bottom	L* Crumb	a* Crumb	b* Crumb
Corn										
Incorporation (I)	Control	73±2	6±1	35±2	67±2	7±1	26±2	76±2	-1.3±0.1	35±2
	Apple	51±2	11±1	25±1	57±3	8±2	24±3	73±2	-0.5±0.2	29±1
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	62±13	8±2	30±5	61±6	9±1	28±1	74±2	-0.9±0.4	34±4
	T3	62±9	9±2	31±6	63±3	6±1	23±3	77±3	-0.8±0.5	28±1
	T5	62±12	8±4	29±4	62±7	8±2	24±1	75±1	-1.3±0.1	30±2
p-value (n=18)	Tukey's HSD test	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Wheat										
Incorporation (I)	Control	69±4	6±1	32±2	66±4	7.5±0.3	29±2	74±1	-0.8±0.4	13±2
	Apple	58±2	10±1	27±1	41±2	12.6±0.5	21±2	66±2	3±1	17±2
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	62±3	7±1	28±2	52±9	10±3	26±4	69±5	1±2	18±1
	T3	62±4	8±3	29±2	52±14	10±2	24±6	72±4	1±2	16.9±0.5
	T5	65±9	9±2	32±3	55±15	10±3	24±2	70±6	1±2	15±1
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	0.001	<0.001	0.007	<0.001	<0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	<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.

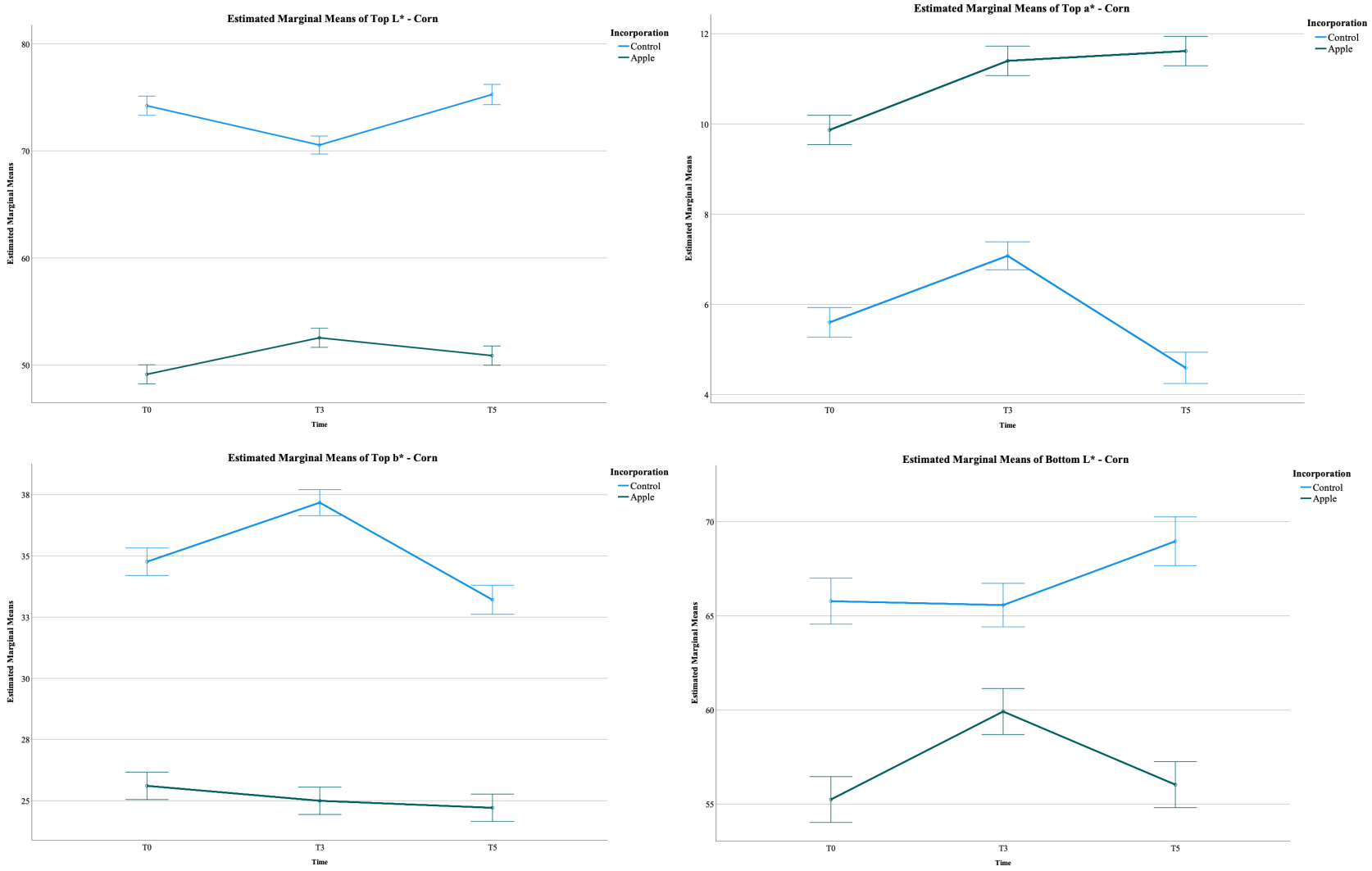

Figure 16. Estimated Marginal Means (EMM) plots for colour coordinates of the top and bottom of the corn bread.

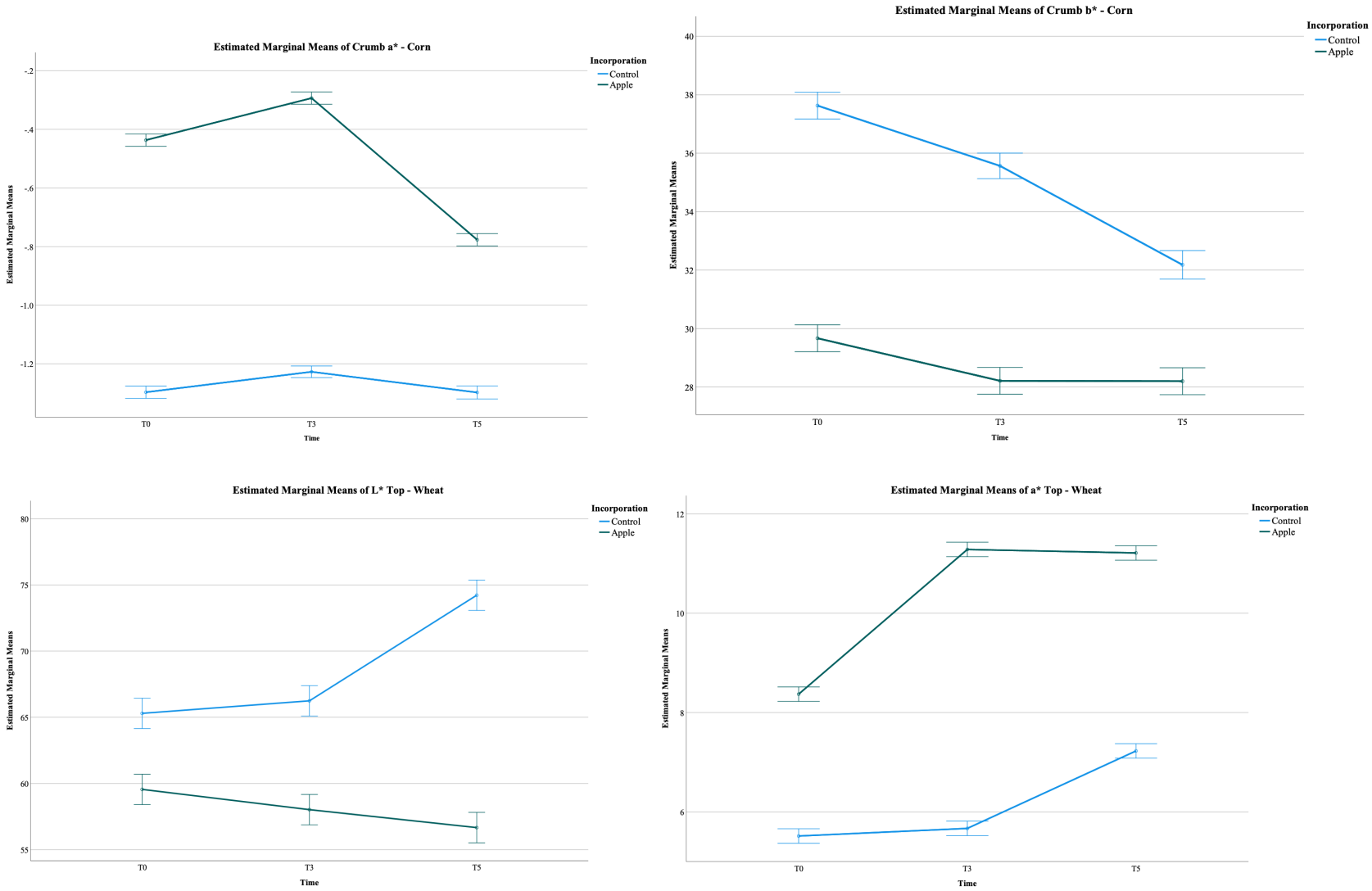

Figure 17. EMM plots for colour coordinates of the crumb of corn bread and top of wheat bread.

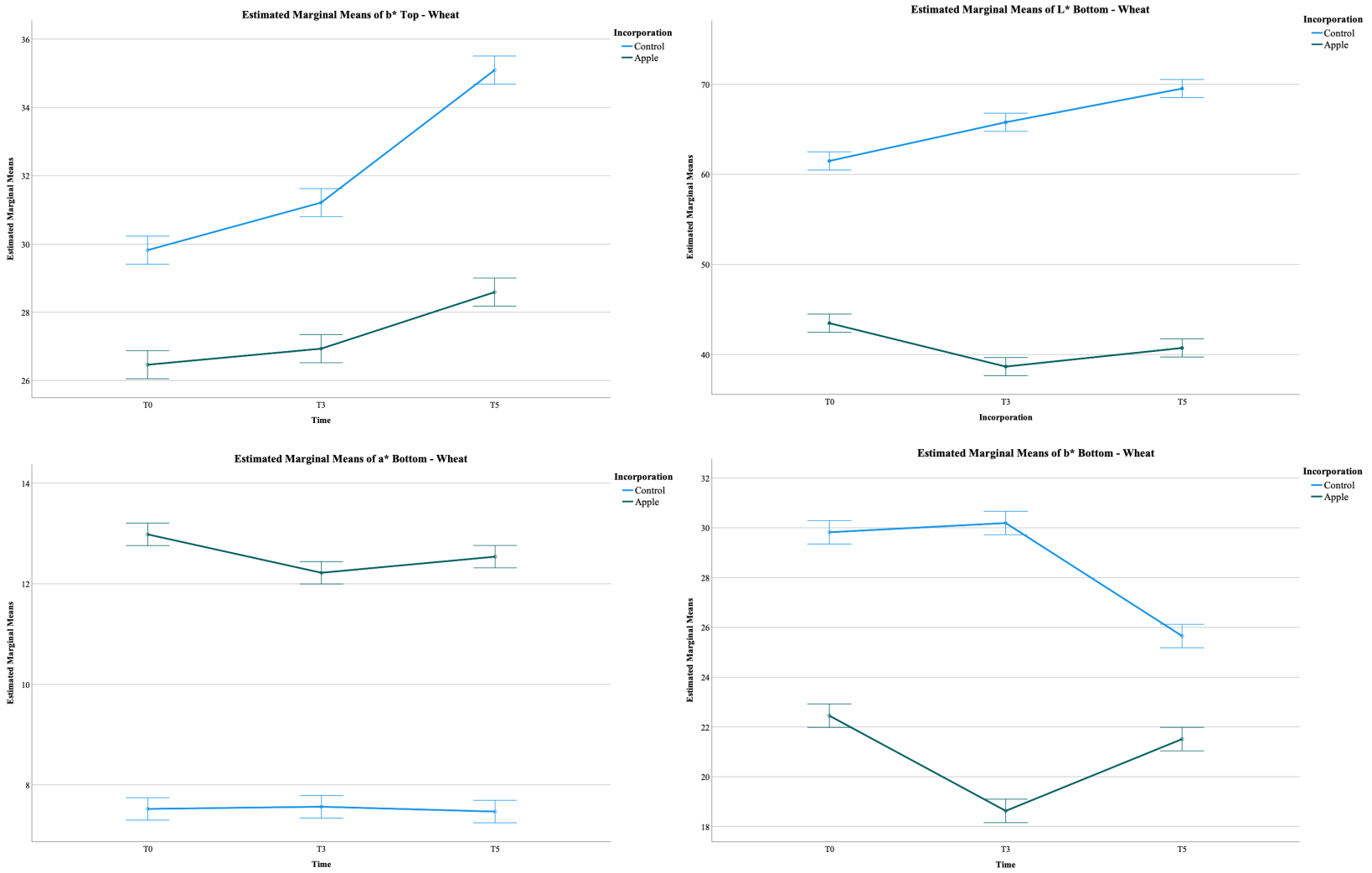

Figure 18. EMM plots for colour coordinates of the top and bottom of wheat bread.

Table 12 shows the six measured dimensions of the breads, namely the hardness (force that teeth have to apply to on the food to deform it), springiness (the rate at which a deformed food reverts to the undeformed state), cohesiveness (the success of a food to withstand a second deformation), chewiness (product of hardness, cohesiveness and springiness, defined as the energy to masticate food) and resilience (similar to springiness, involving the speed and force of the recovery of the food), following a TPA test (Carocho et al., 2020). Considering the low amount of the incorporation of the apple flour, a statistically significant interaction was sought for all the texture dimensions for both bread types. Still, some general tendencies were sought through the EMM plots, shown below.

As detailed above, only some instances of different assays qualify for classification through the estimated marginal means. For the corn bread, the dimensions that allowed for classification were hardness and resilience (**Figure 19**). The EMM plots of **Figure 19** are different from figure **Figure 18**; the XX axis in the former represents the incorporation types while the latter has the storage time. Thus, the XX axis of **Figure 19** should not be regarded as a passage from one to the other and should be regarded as individual points. Regarding hardness, it seems that the highest values was recorded at T3, being the softest of all found at T5. This means that the incorporation of the apple flour did not greatly influence the hardness, which was highly influenced by the passage of time, showing that the bread staling from T3 to T5 could have reduced its hardness, specifically the crispiness of the crust. The highest resilience was found at T0, especially in the control sample, and decreased at T3 and T5. Overall, it seems that the storage time had the highest influence in terms of the texture dimensions in the corn bread, while the incorporation of the apple flour did not show much difference.

The wheat bread allowed classification through the EMM for hardness, springiness and resilience (**Figure 20**). The highest hardness was sought for T5 with T0 and T3 hardly showing differences even between the control and apple incorporated bread. The wheat flour shows different stalling mechanisms and could explain the highest value of hardness at T5. Still, once again the storage time showed higher influence than the incorporation of the apple flour. The EMM plot for springiness shows the progressing over time, in which the control showed a higher springiness than the apple incorporated sample. This is quite desirable, due to the springiness of bread usually being related to its staling, and thus, the apple flour seemed to help reduce this phenomenon. Finally, in terms of resilience, highly influenced by hardness, the T5 bread was the most resilient, although the bread incorporated with flower showed lover values than the control

sample. Overall, once again, the influence of the apple flour was expressed in lower springiness and higher resilience, while the hardness was intensely influenced by the storage time.

Table 12. Texture dimensions of the different breads.

		Corn				
		Hardness (g)	**Springiness (%)**	**Cohesiveness (%)**	**Chewiness**	**Resilience (%)**
Incorporation (I)	Control	20718±12880	0.89±0.04	0.6±0.1	10629±6228	0.4±0.1
	Apple	19288±9774	0.85±0.04	0.5±0.1	9968±5256	0.33±0.04
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	23445±1293	0.91±0.03	0.6±0.1	15260±487	0.5±0.1
	T3	31522±2824	0.85±0.02	0.44±0.02	13100±1787	0.30±0.01
	T5	5042±1339	0.84±0.04	0.62±0.02	2535±540	0.32±0.01
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	0.015	<0.001	<0.001	<0.001
		Wheat				
Incorporation (I)	Control	10589±11746	0.91±0.03	0.7±0.1	5846±6283	0.4±0.1
	Apple	11770±11140	0.85±0.05	0.64±0.03	5443±4807	0.3±0.1
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	2833±511	0.8±0.1	0.7±0.1	1600±51	0.29±0.05
	T3	3670±1164	0.88±0.03	0.66±0.05	2012±649	0.28±0.03
	T5	27034±827	0.90±0.03	0.64±0.03	13321±1320	0.46±0.21
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	<0.001	<0.001	<0.001	<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.

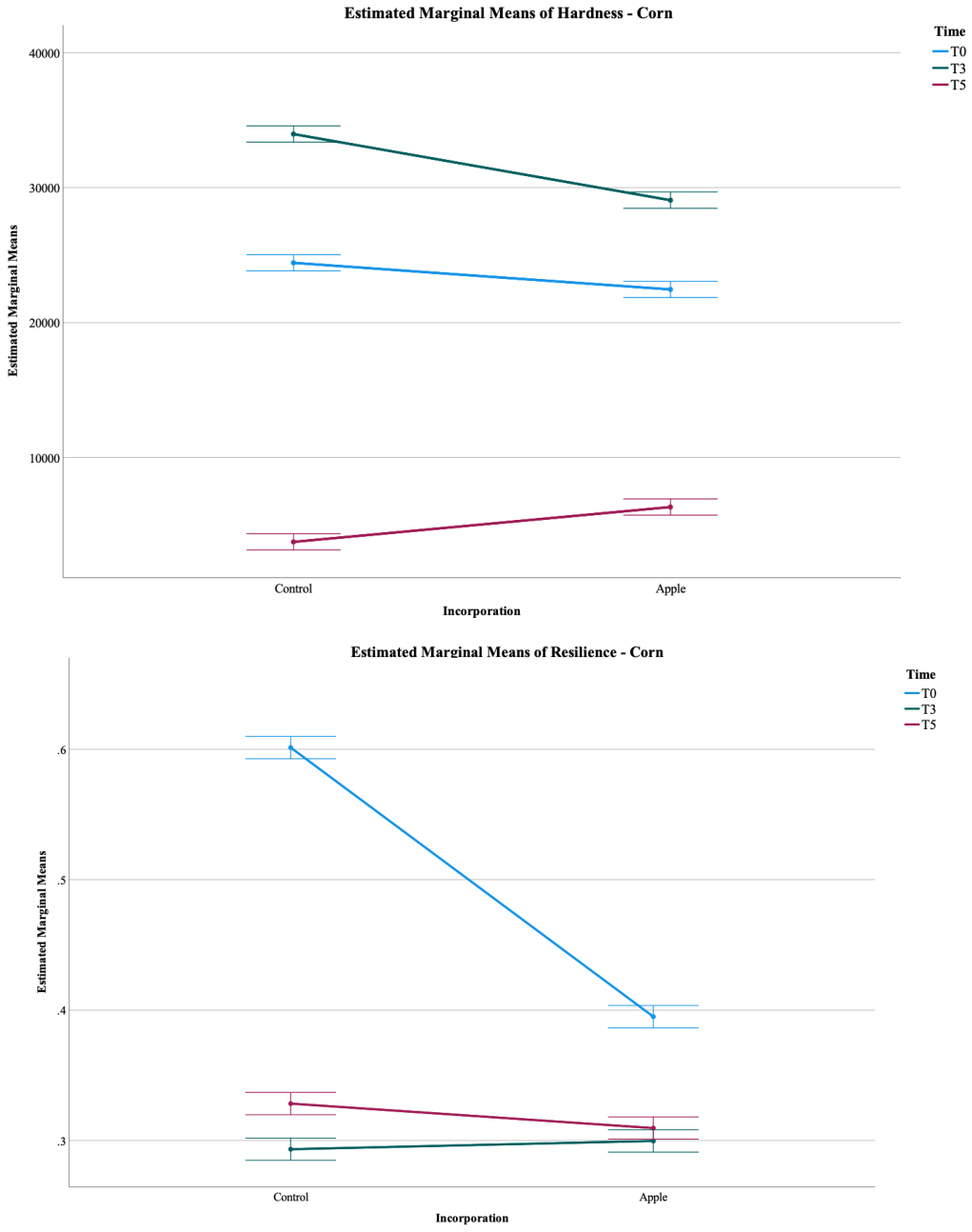

Figure 19. EMM plots for the texture dimensions of the corn bread.

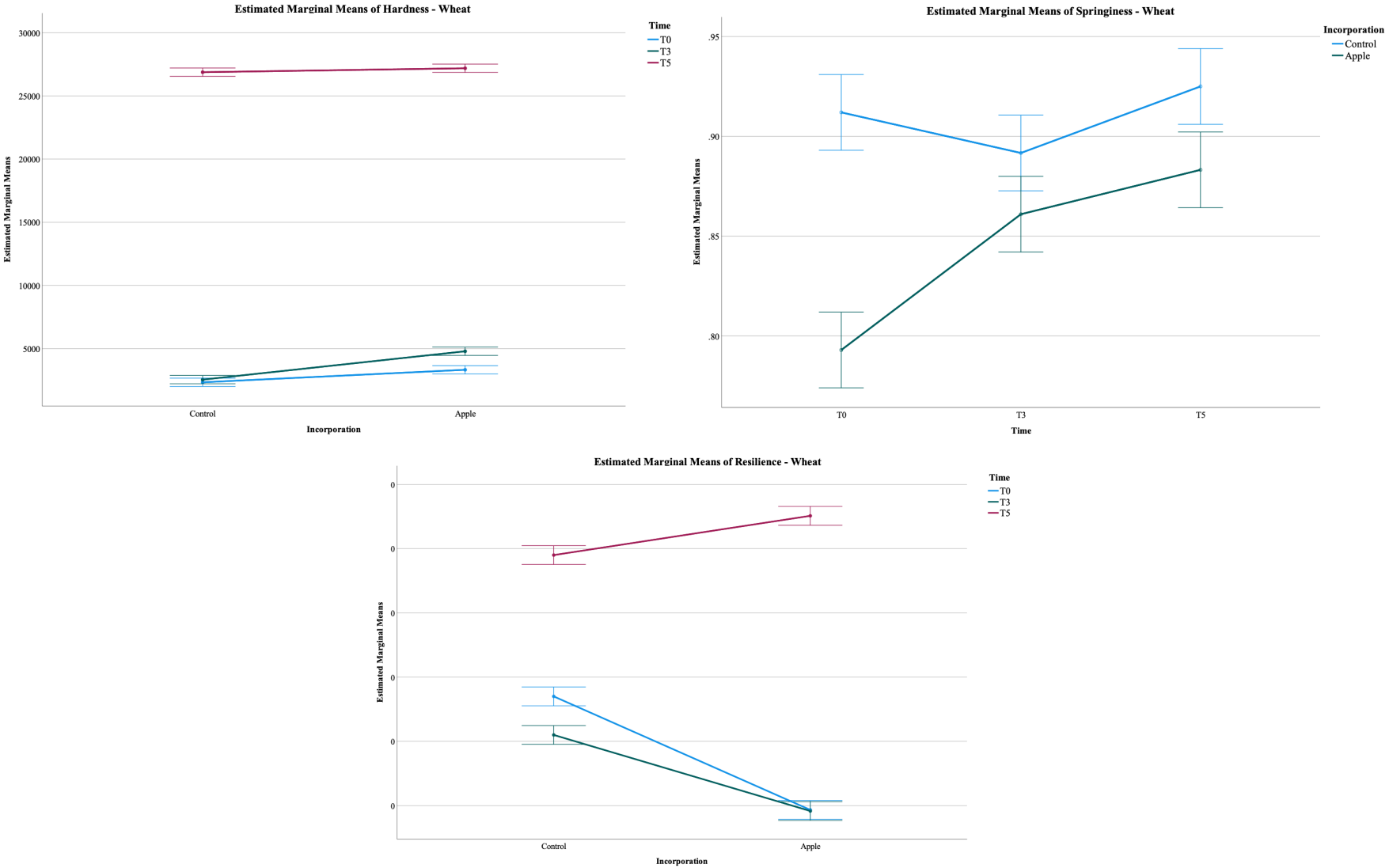

Figure 20. EMM plots for the texture dimensions of the wheat bread.

Table 13. Centesimal compositions of both breads, expressed in g/100g of dry weight.

		Water activity	Humity	Fat	Proteins	Ash	Carbohydrates	Energy (Kcal/100g)	Energy (Kj/100g)
Corn									
Incorporation (I)	Control	0.98±0.01	52±1	0.92±0.02	2.3±0.1	0.70±0.02	44±1	194±3	812±13
	Apple	0.97±0.01	51±2	0.093±0.05	2.2±0.1	0.73±0.02	45±2	197±8	826±33
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	0.97±0.01	52±1	0.90±0.01	2.16±0.04	0.684±0.001	44±1	193±3	807±13
	T3	0.97±0.01	52±1	0.91±0.02	2.3±0.1	0.72±0.02	44±1	193±2	809±10
	T5	0.98±0.01	50±2	0.97±0.04	2.3±0.1	0.73±0.03	46±2	201±7	844±31
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001
Wheat									
Incorporation (I)	Control	0.96±0.01*	43±1	1.65±0.03*	5.8±0.3	1.12±0.03	48±1	231±5	969±20
	Apple	0.92±0.01	37±1	1.86±0.03	5.8±0.1	1.3±0.1	54±1	257±4	1074±15
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	0.95±0.02	41±3	1.6±0.1[a]	5.7±0.2	1.2±0.1	51±3	241±12	1009±50
	T3	0.94±0.02	40±4	1.6±0.1[a]	5.8±0.1	1.3±0.1	51±4	243±16	1017±68
	T5	0.94±0.02	39±3	1.8±0.1[b]	5.9±0.3	1.2±0.1	52±3	248±11	1039±47
p-value (n=18)	Tukey's HSD test	0.06	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	0.79	<0.001	0.099	<0.001	<0.001	<0.001	<0.001	<0.001

In each row, for the storage time different letters mean significant statistical differences, both 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 13 shows the centesimal composition for the two breads, as well as the measured water activity. The results showed a statistically different difference between the control and apple incorporated bread, specifically for the wheat bread, while the storage time did not show difference in this parameter. Still, regarding the wheat bread, statistical differences were sought for fat, in which an increase in fat was found from T3 to T5, but also a higher amount in the apple incorporated bread, meaning that the apple flour had fat that was added to the bread. This nutrient, due to its low amount in wheat showed a significant difference with the addition of the apple flour, although apple does not have a high amount of fat. The increase in fat from T3 to T5 could be justified but the reduction in moisture over time. In terms of the corn bread, all nutrients showed a significant interaction. Moisture, followed by carbohydrates were the most abundant nutrients. Regarding the corn bread no EMM plots were sought.

With regard to the wheat bread, the EMM plots show that the apple flour reduced the overall moisture of the bread while increasing the overall carbohydrates (**Figure 21**).

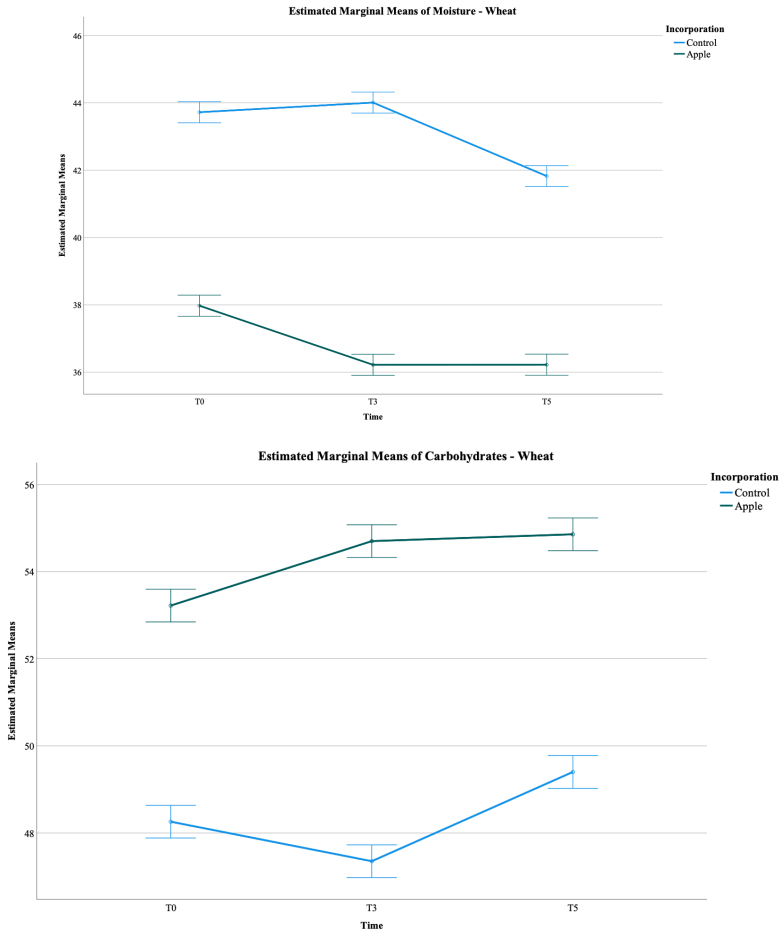

Figure 21. EMM plots for moisture, fat, carbohydrates, and energy for the wheat bread.

Five fatty acids were identified in the breads and represented in **Table 14**, two saturated, one monounsaturated (MUFA) and two polyunsaturated (PUFA). The table also shows the summation of the three different groups of fatty acids. The most abundant individual fatty acid was linoleic acid (C18:2), followed by oleic acid (C18:1). Once again, a significant interaction was sought for all instances of **Table 14**, and so some tendencies were sought from the EMM plots.

For the corn bread, the EMM plots are shown in **Figure 22**, in which it is clear that the apple flour increased the amount of palmitic acid. Regarding MUFA, the control sample showed at T0 the highest value, although over time these fatty acids reduced considerably, while the samples with the apple flour showed maintenance of MUFA. This could be related with the antioxidants in the apple flour which protected these fatty acids over time. For the wheat bread, only one EMM plot could be produced (**Figure 23**). In it, the same profile for MUFA was shown, corroborating the protection of MUFA from the antioxidants of the apple flour.

Overall, the fatty acids profile was not highly impacted by the addition of apple flour, although the MUFA were protect by the antioxidants present in the apple flour, for both bread types.

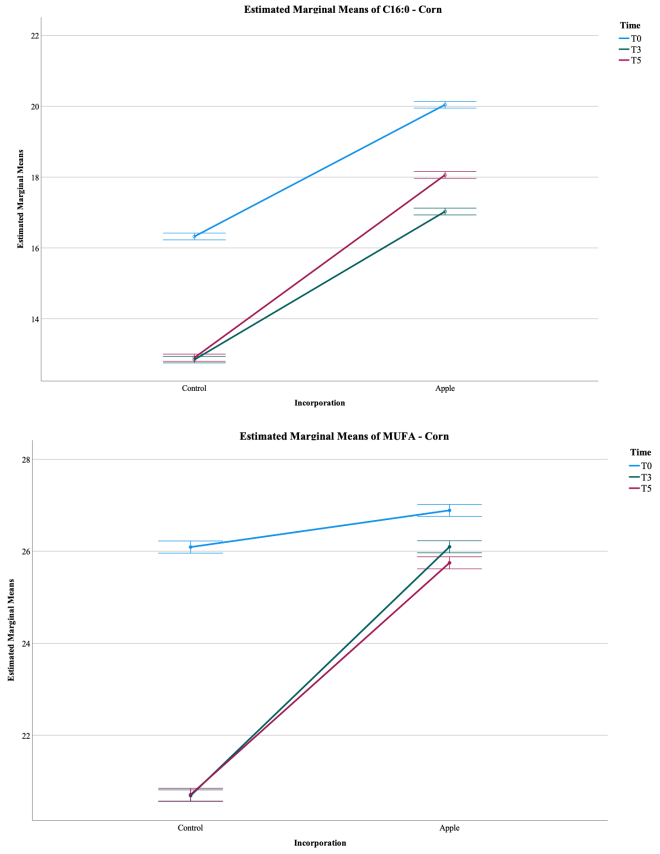

Figure 22. EMM plots for C16:0 and MUFA for the corn bread.

Table 14. Individual fatty acids of the two breads, expressed in relative percentage.

		C16:0	C18:0	C18:1	C18:2	C18:3	SFA	MUFA	PUFA
Corn									
Incorporation (I)	Control	14±2	3.2±0.1	22±3	55±2	6±3	17±2	22±3	60±4
	Apple	18±1	3.0±0.1	26±1	50±2	2.22±0.04	21±1	26±1	52±2
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	18±2	3.0±0.1	26±1	50±2	2.1±0.2	21±2	26±1	52±2
	T3	15±2	3.2±0.1	23±3	54±2	5±3	18±2	23±3	59±5
	T5	16±3	3.13±0.04	23±3	53±3	5±3	19±3	23±3	58±5
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	0.001	<0.001	0.03	<0.001	<0.001	<0.001	<0.001
Wheat									
Incorporation (I)	Control	26±2	3.0±0.4	18.7±0.5	49±2	3.1±0.3	29±2	18.7±0.5	52±3
	Apple	15±1	3.7±0.1	54±4	25±4	2±1	19±1	54±4	27±5
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	20±5	3.1±0.5	38±20	36±15	2±1	23±4	38±20	39±16
	T3	22±7	3.3±0.3	33±15	38±8	3±1	25±7	33±15	41±8
	T5	20±4	3.6±0.2	37±20	37±15	2±1	24±4	37±20	39±16
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<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.

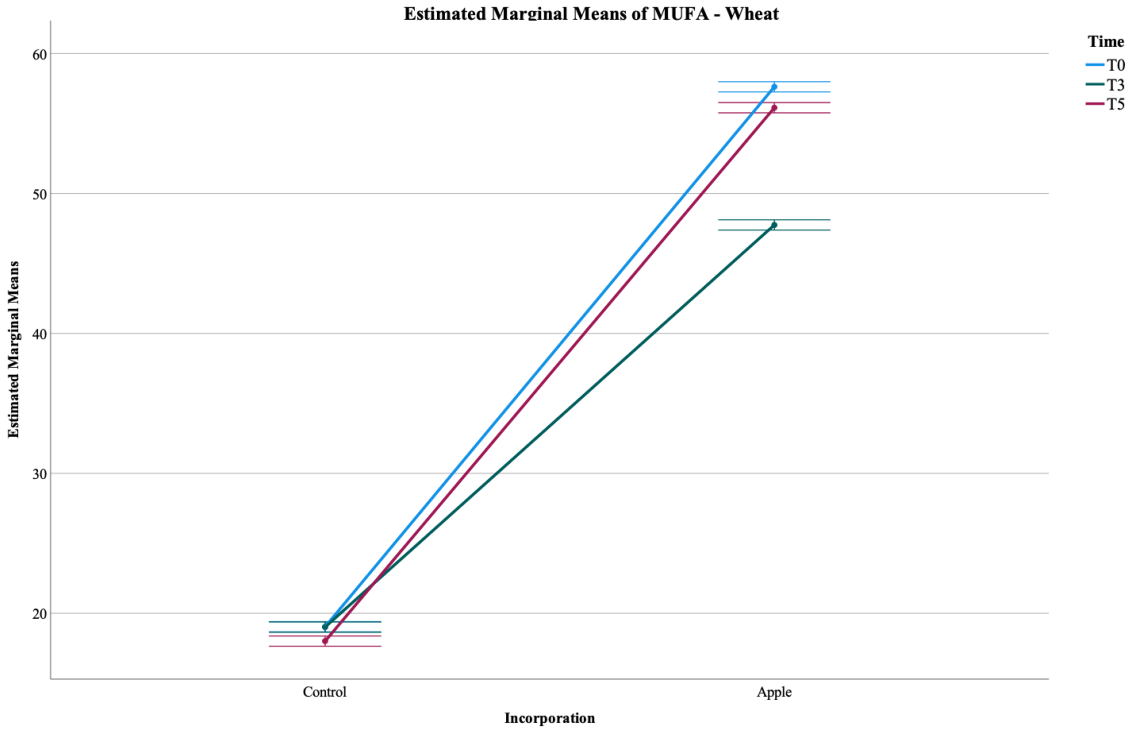

Figure 23. EMM plots for MUFA in the wheat bread.

Table 15 shows the three soluble sugars and their summation found in both breads. Of the three, fructose in the corn bread was the most abundant, while maltose was detected in higher quantities in the wheat bread. Still, among them, a significant interaction was present, hindering statistical classifications, allowing only for general tendencies from the EMM plots.

The EMM plot for the corn bread regarding maltose shows that the addition of apple flour increased the yield in this sugar. The same tendency was recorder for fructose (**Figure 24**). The final plot of **Figure 24** shows the total soluble sugars in wheat flour, showing that the apple flour increased the amount of these three sugars when compared to the control sample. Overall, the apple flour in both breads increased the amount of soluble sugars, although the amount was not very significant, or else a classification could be made.

Table 15. Soluble sugars present in the breads, expressed in mg/mL.

		Corn			
		Frutose	**Glucose**	**Maltose**	**Total**
Incorporation (I)	Control	1.9±0.1	0.86±0.03	0.41±0.02	13.7±0.1
	Apple	2.0±0.2	0.8±0.1	0.42±0.02	13.7±0.5
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	0.142
Storage Time (ST)	T0	2.0±0.1	0.8±0.1	0.44±0.01	13.8±0.1
	T3	1.83±0.03	0.81±0.04	0.39±0.01	13.4±0.3
	T5	2.1±0.2	0.89±0.05	0.42±0.01	14.0±0.4
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	0.444	<0.001
I×ST (n=27)	*p*-value	<0.001	<0.001	<0.001	<0.001
		Wheat			
Incorporation (I)	Control	0.38±0.02	0.23±0.01	4.1±0.1	17±1
	Apple	0.76±0.03	0.26±0.01	5.0±0.2	20.0±0.4
p-value (n=9)	Student T test	<0.001	<0.001	<0.001	<0.001
Storage Time (ST)	T0	0.6±0.2	0.24±0.03	4.4±0.4	18±1
	T3	0.6±0.2	0.24±0.01	4.6±0.4	19±1
	T5	0.6±0.2	0.25±0.01	4.6±0.5	19±2
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	0.001	<0.001
I×ST (n=27)	*p*-value	<0.001	<0.001	0.003	<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.

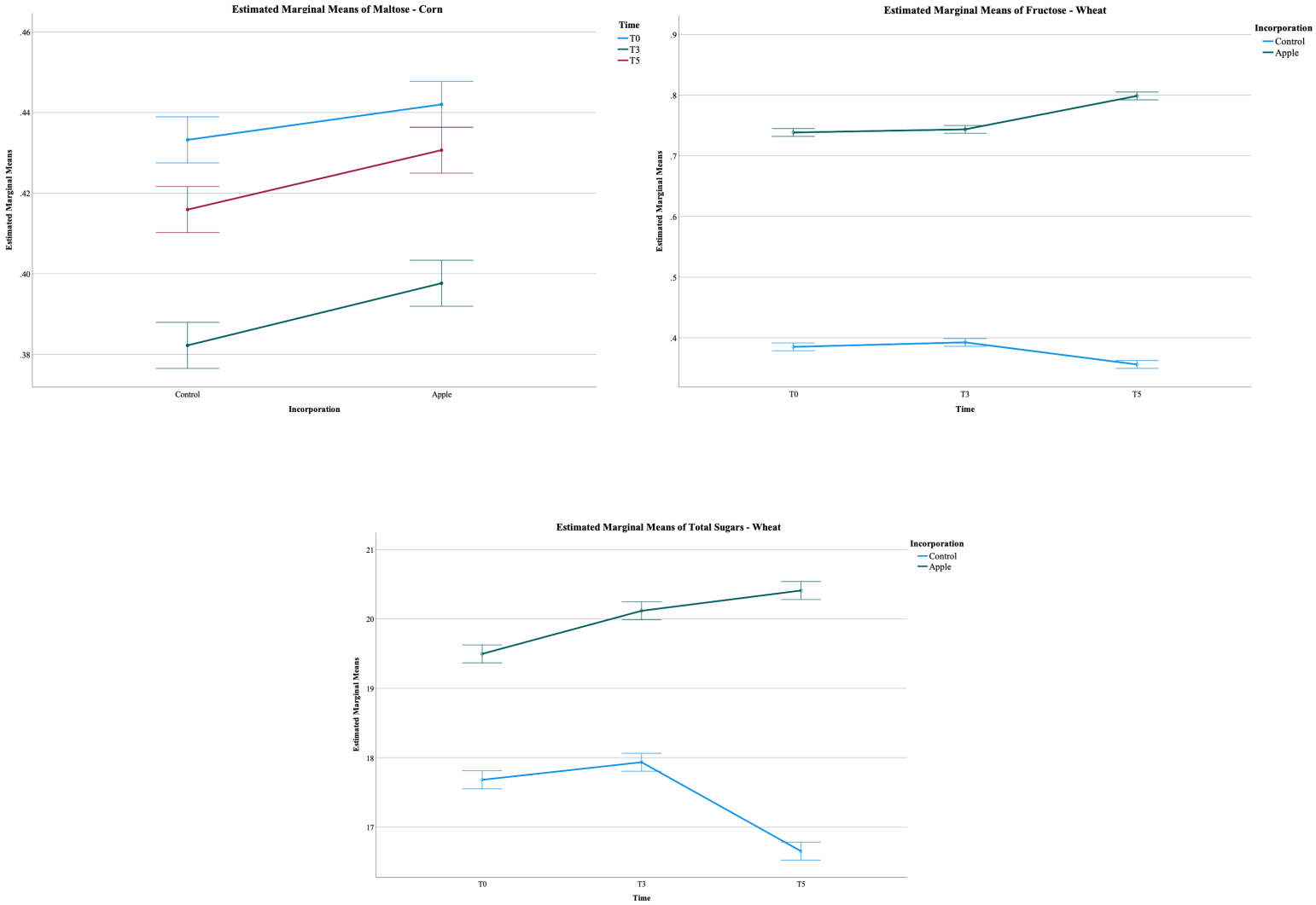

Figure 24. EMM plots of maltose in corn bread and fructose and total sugars in wheat bread.

Table 16 shows the microbial load of the different breads. No contamination for *B. cereus* or yeasts in both breads. Regarding total aerobic mesophiles, for the wheat breads, in interesting feature was sought, namely a statistically significant decrease from T0 to T3 followed by an increase from T3 to T5, while the incorporation with apple at 10% was able to decrease the counting in these microorganisms. Still, higher percentages of apple flour should increase this inhibition of growth. Enterobacteria was found in the control samples of the wheat bread, while no contamination with these microorganisms was found in the samples with apple flour, meaning the antioxidants could avoid their growth.

Table 16. Microbial load of the two bread types, expressed in CFU/mL.

		Corn				
		Total aerobic mesophiles	***Bacillus cereus***	**Enterobacteria**	**Yeasts**	**Molds**
Incorporation (I)	Control	1±1	-	-	-	1±1
	Apple	0.7±1	-	-	-	1±1
p-value (n=9)	Student T test	<0.001	-	-	-	0.422
Storage Time (ST)	T0	2.2±0.2	-	-	-	0±0
	T3	1±1	-	-	-	0±0
	T5	0±0	-	-	-	2.6±0.1
p-value (n=18)	Tukey's HSD test	<0.001	-	-	-	<0.001
I×ST (n=27)	*p*-value	<0.001	-	-	-	0.52
		Wheat				
Incorporation (I)	Control	2±1	-	0.4±0.7	-	1±1
	Apple	2.4±0.3	-	0±0	-	0±0
p-value (n=9)	Student T test	0.544	-	<0.001	-	<0.001
Storage Time (ST)	T0	2.3±0.3[b]	-	1±1	-	0±0
	T3	2.0±0.1[a]	-	0±0	-	0±0
	T5	2.8±0.4[b]	-	0±0	-	1±1
p-value (n=18)	Tukey's HSD test	0.002	-	<0.001	-	<0.001
I×ST (n=27)	*p*-value	0.065	-	<0.001	-	<0.001

In each row, for the storage time different letters mean significant statistical differences, both 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.

A significant interaction was found for both breads in terms of mould growth, thus, some tendencies were extracted from the EMM plots (**Figure 25**). Regarding the TAM (**Figure 25**) in the corn bread, at T0 both samples showed contamination with these microbes, although at T3 they reduced to 0 for the apple incorporated bread, and finally no contamination was found in both breads at T5. This could be due to the lack of favourable environment for these organisms in bread. The moulds found in the wheat samples were only sough in the control samples at T5, meaning the apple antioxidants could hinder the development of these contaminants in the breads.

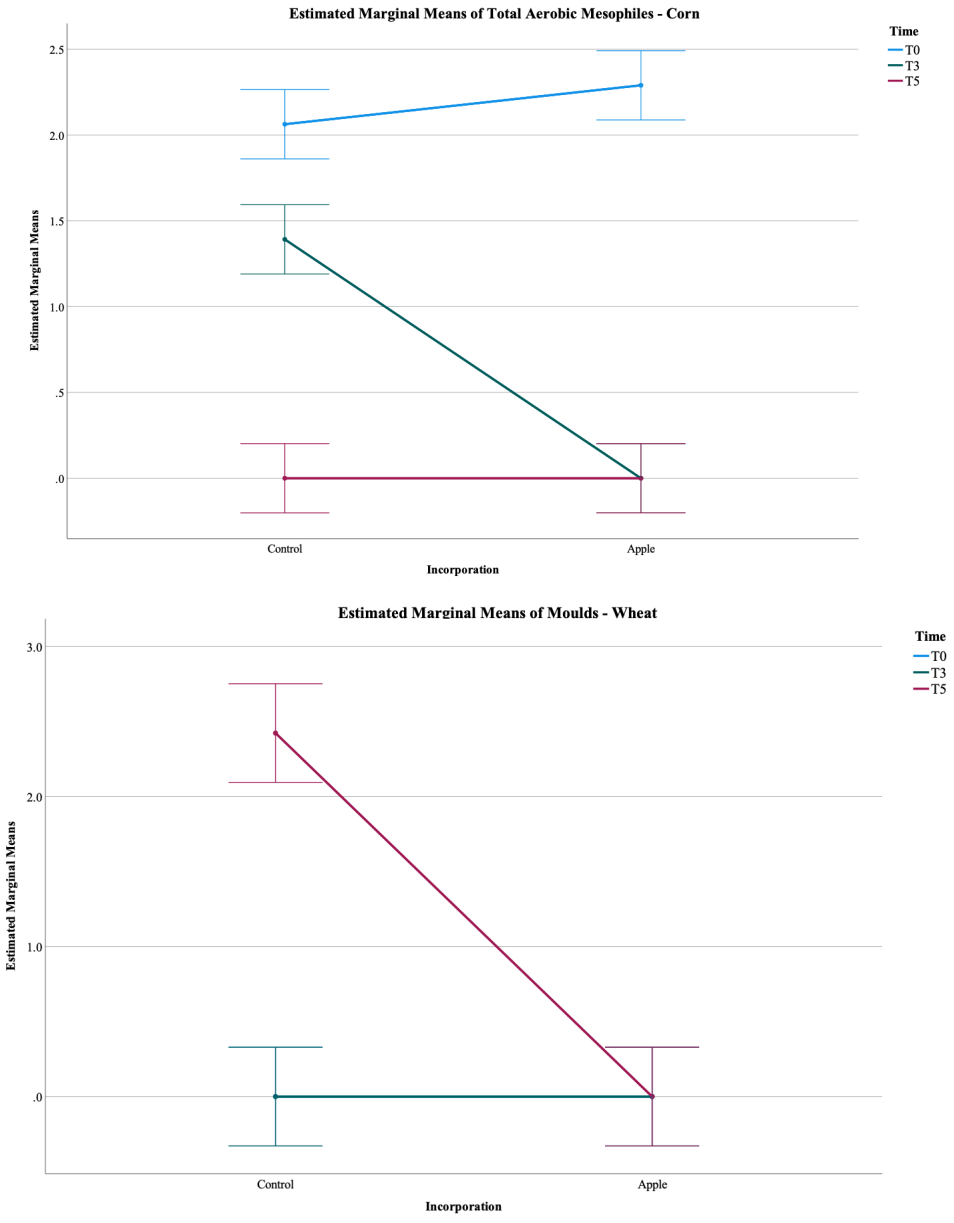

Figure 25. EMM plots of TAM in corn sample, and molds in wheat sample.

Analysing the individual flours, it was possible to see that the apple flour was the one with less counting's in total aerobic mesophiles, suggesting that this flour may have some bioactive compounds that can inhibit these microorganisms. This hypothesis may also justify the inhibition of microorganisms at least until the third day of storage (**Table 17**).

Table 17. Microbial load of the different flours used to prepare the breads, expressed in CFU/mL.

	Corn flour	**Wheat flour**	**Apple flour**
Total aerobic mesophiles	3.3±0.2	4.2±0.2	2.5±0.2
Bacillus cereus	n.d.	n.d.	n.d.
Enterobacteria	n.d.	4.1±0.2	n.d.
Yeasts	n.d.	2.1±0.2	n.d.
Molds	n.d.	2.73±0.05	2.9±0.2

n.d.-not detected

CONCLUSION

Reducing food waste and losses is part of the drivers of the circular economy, which can contribute to fighting pollution and boosting the economy. In this line, it is considered that the process of converting fruit by-products into flour is an economical and viable method for valorization and incorporation into a variety of food products and is a potential source of natural food ingredients. The bakery and pastry industry has been looking for new health-enhancing alternative ingredients as an alternative to traditional ingredients that can recreate new alternative products that can be offered to consumers with some dietary restrictions.

In this sense, the present work aimed to recreate new alternative breads using apple flour obtained from apple bio-waste. Apples are a highly appreciated and consumed fruit worldwide, however, tons of apples are discarded annually because their external characteristics do not correspond to the standard sales characteristics. However, these fruits have a composition rich in bioactive compounds with potential for use that can be exploited at an industrial level.

The apple flour obtained from apples discarded as waste showed a promising chemical and nutritional composition ally with phenolic profile, emphasizing its bioactive potential, which could be explored in different industrial areas, particularly by the food industry as a new ingredient. Apple flour had 3 sugars in its composition, with fructose being the main sugar. Five organic acids were identified from which malic acid appeared in greater quantity. Regarding fatty acids, there was a prevalence of MUFAs, mainly due to the contribution of oleic acid. The phenolic characterization of apple flour revealed the presence of seven phenolic compounds, highlighting the prevalence of phenolic acids. Regarding the bioactivities, the study of the antioxidant activity carried out through four in vitro assays revealed that apple flour showed antioxidant and antimicrobial activity but only revealed anti-proliferative activity for one of the tumor cell lines studied.

The addition of apple flour in wheat and corn bread seems to result in a new and interesting proposal for alternative bakery products. The addition of apple flour in a reduced percentage of 10%, allowed to guarantee the texture and traditional nutritional composition respecting the original characteristics of this product so appreciated by the consumer. The colour seems to have been slightly improved which is always well accepted by the bakery industry which guarantees to be an attractive feature in a first impact of the product. Regarding the fatty acid composition, the incorporation does not seem to cause significant changes,

however, the antioxidants present in apple flour seem to guarantee protection in the MUFA present. The evaluation of the microbial load suggests that the apple flour seems to hinder the growth of contaminants in the breads.

Thus, with this study, it was found that it is possible to reuse the apple by-products resulting from the agri-food industry for the development of a potential value-added food ingredient (apple flour), and subsequent incorporation into bakery products. By reusing this type of by-product, the amount of waste that is directly eliminated into the environment is also reduced, contributing to the reduction of environmental pollution and food waste.

Apple by-products are a good source of functional compounds that can be reused for nutritional enrichment of other products. Since these constituents have health benefits, the commercial exploitation of apple by-products for the recovery of these compounds seems to be interesting.

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