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# THÈSE

## EN VUE DE L'OBTENTION DU DIPLOME DE DOCTORAT

**Domaine : Sciences de la Nature et de la Vie.** **Filière : Biologie**
**Spécialité : Microbiologie Appliquée**

**Présentée par**
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***Thème***

# Formulation d'un lait fermenté synbiotique à base de souches probiotiques locales et de poudre de caroube

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## THESIS

## SUBMITTED TO OBTAIN THE DOCTOR DEGREE

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**Presented by**

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***Thesis title***

# Formulation of a synbiotic fermented milk using local probiotic strains and carob powder

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# Dedication

**“I do thank Allah for all gifts which given me”**

I dedicate this thesis to,

My beloved Yemma, who I cannot thank enough for the sacrifice and support she has shown throughout my life and academic career. I know that my workload has often kept me from you and my family;however, you never stopped supporting me even when I was absent.

My beloved husband, for his endless patience and unwavering support; this thesis was a chapter of our lives which proved challenging, I am eternally in your debt for being there with me through it all.

All members of my family for their support;

***“Everything is possible. The impossible just takes longer.”***

***- Dan Brown***

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## List of abbreviations

**ABTS**: 2,2'-azino-bis (3-éthylbenzothiazoline-6-sulphonique) acid

**ANOVA**: One-way analysis of variance

**ATCC**: American Type Culture Collection

***Bf***: *Bifidobacterium*

**BHI**: Brain Heart Infusion

**CFSs**: Cell-Free Supernatants

**CFU**: Colony Forming Unit

**CVD**: Cardio-Vascular Disease

**DMEM**: Dulbecco's Modified Eagle Medium

**DNS**: 3, 5-DiNitro Salicylate

**DP**: Degree of Polymerization

**DPPH**: 2,2-diphenyl-1-picrylhydrazyl

**FAO:** Food and Agricultural Organization

**FGIDs**: Functional Gastrointestinal Disorders

**FOS**: FructoOligoSaccharides

**GAE**: Gallic Acid Equivalent

**GIT**: Gastro-Intestinal Tract

**GOS**: GalactoOligoSaccharides

**GRAS:** Generally Regarded As Safe

**HPLC**: High Performance Liquid Chromatography

**IBD**: Inflammatory Bowel Disease

**IBS**: Irritable Bowel Syndrome

**Ig**: Immunoglobulin

**ISAPP**: International Scientific Association for Probiotics and Prebiotics

**Kat**: Katal

**LAB:** Lactic Acid Bacteria

***Lb***: *Lactobacillus*

***Lc***: *Lactococcus*

**MRS**: de Man, Rogosa and Sharpe

**ORAC**: Oxygen Radical Absorbance Capacity

**PBS**: Phosphate Buffered Saline

**PDA**: Photodiode Array Detector

**RF**: Residue Fraction

**RP-HPLC**: Reverse-phase High Performance Liquid Chromatography

**RUE**: Rutin Equivalent

**SGF**: Simulated Gastric Fluid

**SIF**: Simulated Intestinal Fluid

**SM**: Skim Milk

**spp** : Species plural

**SSF**: Simulated Salivary Fluid

***Str***: *Streptococcus*

**T2D** : Type 2 Diabete

**TA**: Titrable Acidity

**TE**: Trolox Equivalent

**TFC**: Total Flavonoid Content

**TPC**: Total Phenolic Content

**WHO:** World Health Organization

**XOS**: XyloOligoSaccharides

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## General introduction

Over the last decade, consumers indicate an increasing desire for novel and "healthy" foods with improved sensorial, nutritional and functional criteria. Nowadays, the advances in scientific research support the idea that "functional foods" may fulfill nutritional needs and exert a beneficial role in some diseases (Otles &Cagindi, 2012).Among the large number of novel and innovative functional foods under investigation or alreadypresent in the market, fermented dairy products including fermented milks are considered the most promising category (Corbo *et al.*, 2014).The importance of fermented milks in human diet is well established even if their role has been evolved in the time.The microbial community in milkdrives the fermentation process releasing bioactive components that confer nutritional, safety, organoleptic and health beneficial properties to the final product(Marco *et al.*, 2017).

Different fermented milks are produced using probiotic bacteria(Barat &Ozcan, 2018;Temiz &Çakmak, 2018). The use of probioticmicroorganisms (of the genera*Lactobacillus*and*Bifidobacterium*)infermented milks has also been a way of differentiating the product.These microorganisms are known to provide several health benefits, primarily by maintaining the balance of the gastrointestinal tract (Champagne*et al.*, 2018; Fazilah*et al.*, 2018). In order to improve the action of probiotics and increase fiber ingestion, prebiotics have also been successfully incorporated in a variety of dairy products (Santos *et al.*, 2018), such as yogurt (Marinaki *et al.*, 2016), fermented milk (Canella *et al.*, 2018), Greek yogurt (Costa *et al.*, 2019), whey beverage (Souza *et al.*, 2019) and mousse (Xavier-Santos*et al.*, 2019). The prebiotic has been defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson *et al.*, 2017). Because of the synergistic potential between probiotics and prebiotics, the combination there of is considered "synbiotic" and may benefit the host due to the increased survival of the microorganisms in the gastrointestinal tract (Iraporda*et al.*, 2019). Thus, developing novel synbiotic fermented milk is an alternative to supply the growing demand from consumers who long for functional dairy products.

Therefore, the screening for new functional probiotics strains is a very valuable tool to maintain biodiversity and to use un/less exploited sources. Fermented foods obtained from the spontaneous activity of the autochthonous microbiota, as well as autochthonous Lactic Acid

Bacteria (LAB), present in raw vegetable or animal feedstock (Motato *et al.*, 2017; Tamang*et al.*, 2016), still remain a natural and rich reservoir of biodiversity to search for “novel” strains with great technological and functional properties.

In Algeria traditional fermented dairy products, following artisanal practices, have been consumed for a long time in relation to ethnic Berber traditions. Cheeses (“Klila”, “Bouhezza”, “Kamaria”, and “Jben”), fermented milks (“Lben” and “Raib”) and some fat-based products(“Smen” and butter) are mostly homemade in the rural areas of Algeria (Bendimerad*et al.*, 2012; Mechai *et al*., 2014; Medjoudj*et al.*, 2018). They can be used as a potential of probiotics as they commonly contain LAB. Unfortunately, the microbial community of Algerian dairy products is not well characterized except few works which focused on the characterization of the autochthonous LAB (Mechai *et al*., 2014; Bachtarzia *et al*., 2019).

In another hand, there is great economic interest in finding other prebiotic-rich food matrices. The nutritional composition of Carob which includes complex polyphenols and functional dietary fibers (Owen *et al.*, 2003; Rakib *et al.*, 2010; Uysal *et al.*, 2016) could make this ingredientvery good sources of prebiotic components for human nutrition andprobiotic bacteria, besides being also alow-cost ingredient. The interest in polyphenols is mainly attributed to their contribution to human health through multiple biological effects which includes antioxidant, anti-inflammatory, anti-mutagenic and anti-carcinogenic activities (Fraga*et al.*, 2010; Kim *et al.*, 2014).The carob production in Algeria is important but its industrial use and exploitation is very low, where the produced quantities are exported to other countries in most cases. To the best of our knowledge, the use of carob powder as a potential prebiotic ingredient in probiotic fermented milk has not been considered yet in Algeria.

Thus, the main purpose of this work was to study the feasibility of developingfunctional synbiotic fermented milk using local starter and probiotic LAB strains isolated from Algerian artisanal cheeses and pulp carob powder as a prebiotic ingredient to enrich the Algerian dairy industry with a novel product. The specific aims of the study were:

1. Isolation, identification and characterization of starter and probiotic LAB strains from Algerian artisanal cheeses.

2. Study of the carob powder's polyphenols composition and their changes under a simulated gastrointestinal digestion as well as their biological activities.
3. Incorporation of carob powder into a probiotic fermented milk.
4. Study of the composition and the shelf-life of the formulated synbiotic fermented milk during storage and during a simulated gastrointestinal digestion, and its biological activities.

**Part 1: Literature review**

# 1 Probiotic

## 1.1 Concept and definitions

The probiotic concept was proposed in 1908 by Russian Nobel laureate Elie Metchnikoff who, observed that regular consumption ofLAB fermented dairy products, elicited beneficial effects on human health promoting longevity in elders of Bulgarian people (Anukam&Reid, 2007). Since then, the term "probiotic" has been linked to beneficial bacteria for health promotion, although its precise definition has evolved over the time.

The initial definition of probiotics was proposed as early as in 1965. Subsequently, the FAO/WHO defined probiotics as "live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). According to the descriptions from International Scientific Association for Probiotics and Prebiotics (ISAPP), the spectrum of products that can be classified as probiotics comprises not only beneficial bacteria, but also others. These include drugs and enteral feedings for amelioration of diseases, food supplements for promotion of the benefits of health, infant formula such as the milk powders, and even the animal feedings (Hill *et al.,* 2014).The underlying mechanisms on how and why the bacterial strain(s) act to achieve such effects have been under intensive study (Nagpal *et al.,* 2012). In general, it is not necessary that probiotics colonize the target organ such as the intestine. However, at least certain number of live bacteria have to reach the colon where they can affect the local intestinal ecology, physiology and metabolisms (Bourlioux *et al.*, 2003). By definition, probiotics should be safe in animal, resistant to acidity and bile acids, and able to adhere and colonize the intestine (Papadimitriou *et al.*, 2015).

Nowadays, there are many different species of probiotics widely used. The most common are belonging to *Bifidobacterium* and *Lactobacillus*genera, but also can be other LAB such as lactococci and streptococci. Other promising probiotic strains include organisms of the genera *Bacillus*, *Enterococcus*, *Escherichia*, *Propionibacterium*, and the yeast genus *Saccharomyces* (Table 1).

**Table 1.**Examples of microorganisms that are considered to be probiotics (Fijan, 2014)

| ***Lactobacillus* spp.** | ***Bifidobacterium* spp.** | **Others** |
|---|---|---|
| *Lb. acidophilus* | *Bf. bifidum* | *Escherichia coli* Nissle |
| *Lb. casei* | *Bf. breve* | *Saccharomyces boulardii* |
| *Lb. crispatus* | *Bf. infantis* | *Streptococcus thermophilus* |
| *Lb. delbrueckii* subsp. *bulgaricus* | *Bf. longum* | *Enterococcus faecium* |
| *Lb. fermentum* | *Bf. lactis* | |
| *Lb. gasseri* | *Bf. adolescentis* | |
| *Lb. johnsonii* | | |
| *Lb. paracasei* | | |
| *Lb. plantarum* | | |
| *Lb. reuteri* | | |
| *Lb. rhamnosus* | | |

*Lb.*:*Lacticaseibacillus*,*Bf.*:*Bifidobacterium*

### 1.2 Important properties for effective probiotics

The proper characterizationof a probiotic is an important procedure to meet the desired health effect. Probiotics have different origin and principally they are from human large and smallintestines, or breast milk, animal origin, food sources like raw milk or fermented foods(Mercenier *et al.*, 2008).

To be active, a potential probiotic is expected to reach the site, where it is presumed, in a good amount. For maximum activity, the strain should be able to proliferate and colonize at this specific location. Besides, it should also be tolerated by the immune system. It should not be pathogenic, allergic, or mutagenic/carcinogenic (Ohashi & Ushida, 2009). Probiotics for humans should have 'generally regarded as safe' (GRAS) status, with a proven low risk of inducing or being associated with the etiology of disease. The probiotic organisms should preferably be of human origin, must be able to survive and grow in the *in vivo* conditions of the desired site of administration, and thus must be able to tolerate low pH and high concentration of both conjugated and deconjugated bile salts. For successful application in foods, the probiotic used should also be technologically compatible with the food manufacturing process. In addition to that, the foods containing the probiotic bacteria must maintain the characteristic sensory attributes of the traditional food(Nagpal *et al.*, 2012).

## 1.3 Beneficial health effects of probiotics and their mechanisms of action

There is increasing evidence in favour of the claims of beneficial effects attributed to probiotics.However, the functions of these probiotics vary significantly within the same species, mostly up to and dependent on some specific strains. Thus, in evaluating the functions of the probiotics, it is essential to characterize the functions of each probiotic to the specific strain. Possible mechanisms of action may include: (1) enhancing the natural barrier function of the normal intestinal mucosa, (2) modulation of the immune system (3), antagonism of pathogens and (4) production of enzymatic activities and/or beneficial metabolites for the host (McFarland, 2009) (figure 1).

- **Antagonistic action against pathogenic microorganisms**

The most important action of a probiotic is unquestionably the protection against infection and colonisation of the digestive tract by pathogenic microorganisms. The mechanisms that form the host's first line of defence against intestinal infection are called resistance to colonisation, competitive exclusion and the barrier (Linares *et al*., 2016)effect. Pathogenic microorganisms can be suppressed in several ways (Linares *et al.*, 2016; Tsiouris & Tsiouri, 2017; Sotoudegan *et al.,* 2019):

• Acidification of the digestive tract by organic acids (e.g. lactic or acetic acid) production.

• Production of bacteriocins, antimicrobial substances that inhibit the pathogens.

• Interaction with mucosal and epithelial surfaces, enabling pathogens adhesion and preventing their colonisation.

- **Stimulation of immunity**

Probiotic strains have a stimulating action on the host's immune system, acting both on the cells involved in natural immunity and on those related to specific immunity, and also activating macrophages. Although the full mechanisms have not yet been elucidated, it is known that only microorganisms capable of surviving in the gastrointestinal (GI) tract can activate macrophages. In addition, it seems that the presence of probiotic microorganisms favours antibody production, especially secretory immunoglobulin A (IgA) in the intestinal lumen, which can inhibit the adherence of pathogenic bacteria to the mucosal surface: • Causing the

agglutination of bacteria. • Modifying the adhesion factors present on the surface of the bacteria. • Interfering with adhesin-receptor interactions. Due to their action on the immune system, LAB have the potential to prevent intestinal infections, protect against damage related to the immune system and act as immunomodulators (Cerbo*et al.*, 2016; Mishra& Mishra, 2018).

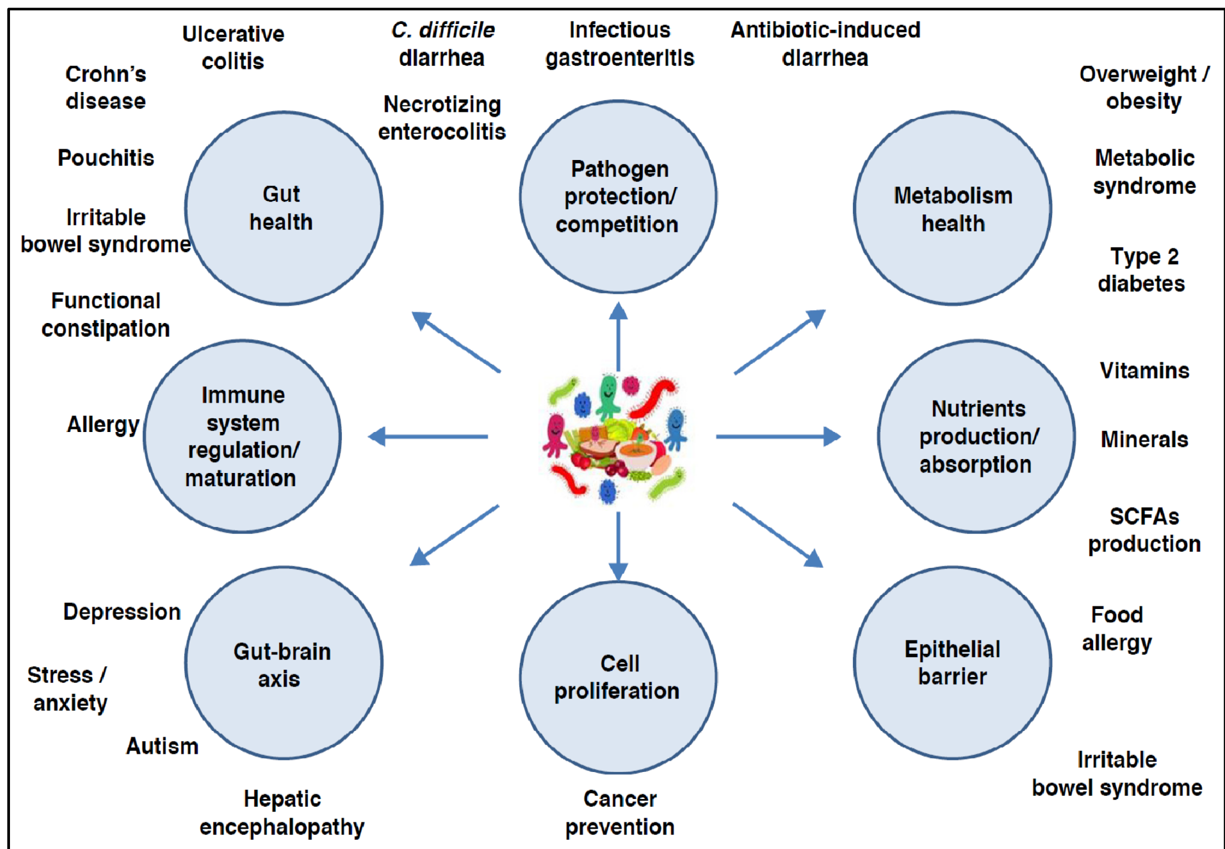


**Fig. 1:** Schematic representation of the main mechanisms of action through which probiotics and prebiotics exert their health effects (adapted from Cremon *et al.,* 2018). SCFAs: Short Chain Fatty Acids; *C. difficile*: *Clostridium difficile*

➢ **Neutralisation of toxic products**

Inactivation of toxic compounds is another very important aspect of probiotic action. It seems that probiotics attenuate intra digestive catabolism, orienting liver function. They accumulate in the gut microbiota where they reduce the absorption of toxic substances such as ammonia, amines and indole. It also seems that they reduce the biotransformation of bile salts and fatty acids into toxic products (Sotoudegan *et al.,* 2019).

- **Modulation of stress**

Stress is one of the factors that influence variations in the gut microbiota. Stress alters digestive physiology, increasing peristalsis and secretions of HCl and mucus in the digestive tract, and thus modifying the microbiota and the activities that depend on it (Novik & Savich, 2019).

- **Protection of the urogenital system**

In healthy women, the urogenital system is characterised by a complex microbiota whose equilibrium undergoes numerous fluctuations. Multiple studies have confirmed that endogenous lactobacilli play a similar role in the prevention of infection in the urogenital system as they do in the intestine (Cerbo *et al*., 2016).

- **Bacterial overgrowth, intestinal motility disorders and intestinal microbiota**

Bacterial overgrowth syndrome is defined as abnormal bacterial proliferation in the small intestine, generally due to the previous existence of anatomical alterations or poor intestinal motility. In most cases, it only causes mild nonspecific symptoms such as prolonged diarrhoea, flatulence and abdominal pain. However, bacteria can damage the intestinal mucosa, leading to malabsorption syndrome which in turn leads to secondary malnutrition due to loss of nutrients. Overgrowth of Gram-negative bacteria in the intestinal lumen displaces the normal microbiota of the small intestine, giving rise to a series of effects that are responsible for malabsorption symptoms. Studies of probiotic administration as adjuvant treatment constitute a promising therapeutic approach in this field (Sotoudegan *et al.,* 2019).

- **Implication and effects of probiotics in different diseases**

Increasing numbers of studies have analysed intestinal microbiota variability in different inflammatory diseases of the intestine such as coeliac disease (de Sousa Moraes*et al*., 2014) and Crohn's disease (Gensollen & Blumberg, 2017). Effective modification of the gut microbiota is therefore considered a promising therapeutic approach that influences the immune response. Probiotics play an important role in modulating intestinal lymphoid tissue and exert an immunomodulatory effect; consequently, they may have a therapeutic application in some autoimmune diseases or as prophylactics (de Sousa Moraes *et al*., 2014).

## 1.4 *Lactobacillus brevis* and its probiotic potentialities

*Lactobacillus (Lb.) brevis*is an heterofermentative LAB isolated from milk, cheese, sauer-kraut, sour dough, silage, cow manure, faeces, mouthand intestinal tract of humans (Rönkä *et al.*, 2003). As a member of the genus*Lactobacillus*and due to its long-term use in various traditionallyfermented food products,*Lb. brevis*has the GRASstatus.*Lb. brevis* was not, typically consideredas a probiotic. However, O'Sullivan *et al.* (1992)andCollins *et al.* (1998)have mentioned*Lb. brevis*in alist of strains that can be used in probiotic products. Since that, several studies have been conducted on *Lb. brevis* strains especially those isolated from traditional fermented foods for their probiotic potential.

*Lactobacillus brevis* KB290, isolated from suguki, a traditional Japanese fermented pickle was reported to tolerate digestive juices, stimulates immune function (Kishi *et al.,* 1996), and improves gut health (Nobuta *et al.,* 2009). This strain meets the criteria for a probiotic and it is safe for human consumption (Yakabe *et al*., 2009). It was also reported that KB290 plays a crucial role in people with infuenza and suppress inflammation caused by nitric oxide (Abdelazez *et al.,* 2018).

Rönkä *et al*. (2003) have reported that *Lb. brevis*ATCC 8287 exhibited good *in vitro* adherence to human Caco-2 cells, tolerance to gastrointestinal digestion and inhibition properties toward selected potential harmful microorganisms, particularly against *Bacillus cereus*. Also, this strain was detected in the faeces following examination of its survival through human intestine after digestion. Similarly, *Lb. brevis* KU15006, a strain isolated from South Korean homemade kimchi, was reported to fulfill the criteria for use as a probiotic, including acid and bile salt tolerance, enzyme activity profile, intestinal cell adhesion, and antibiotic susceptibility. In addition, *Lb. brevis* KU15006 inhibited the adhesion of *E. coli* and *Salmonella enterica*Typhimurium to HT-29 cells, and its cell free supernatant presented higher inhibition of α-glucosidase than those of the commercial strains (Son *et al.,* 2017).

Another study of Aarti *et al.* (2017) group investigated the probiotic potential of *Lb. brevis*LAP2, isolated from *Hentak*, a fermented dried fish food in India. Strain LAP2 depicted not only antibacterial characteristics against human pathogens and huge range of functional probiotic characteristics, but also antioxidant properties by scavenging the radical DPPH (2,2-

diphenyl-1-picrylhydrazyl) and exhibiting significant resistancetowards various concentrations of hydrogen peroxide which, might be a potential candidate for reducing the oxidative stress associated diseases in humans.

Furthermore, *Lb. brevis* KU200019 isolated from Jeotgal, Korean fermented seafood, showed dominant probiotic properties compared to commercial strains such as higher survival rate in gastric conditions and antimicrobial activity against various foodborne pathogens. Moreover, synergistic interactions between *Lb. brevis* KU200019 and FOS (Fructooligosaccharides) markedly enhanced the inhibition of foodborne pathogens adherence to HT-29 cells and confirmedits potential use in modulationof the gut microbiota and prevention of pathogens-associated diarrhea. Furthermore, high survival rate over 8 log CFU/mL in skim milk and high antioxidant activity in fermented skim milk confirmed the potential use of *Lb. brevis* KU200019 as an adjunct culture in synbiotic-fermented dairy products to enhance their safety and quality (Kariyawasam *et al.,* 2020).

All these studies represent some examples among many others that show the potentialities of *Lb. brevis*to be a good candidate for probiotic use in fermented foods.

## 2 Prebiotics

### 2.1 Definition

In the 1980s, it was postulated that some components ofthe diet could promote the growth ofcertain bacterialstrains present in the intestine which, are closelyassociated with benefits for host health(Janssen & Kersten, 2015). Subsequently, the term “prebiotic” was generally accepted and defined by Gibson and Roberfroid asfood ingredients that are non-digestible and show beneficial effects on the host by stimulating the growth and/or activity of one or alimited number of bacteria in the colon, thus improvingthe host health(Slavin, 2013). Because ofthe fact that prebiotics are not the only substances thatmay affect gut microenvironment, the selectivity of the prebiotic fermentation differentiates them from other undigesteddietary ingredients and compounds, such as antibiotics,minerals, and vitamins(Gibson *et al.*, 2017).

An ideal prebiotic should be **(1)** resistant to the action of acids in the stomach, bile salts and other hydrolysing enzymes in the intestine, **(2)** should not beabsorbed in the upper gastrointestinal tract, and **(3)** should be easily fermentable by thebeneficial intestinal microflora(Kuo, 2013).

## 2.2 Sources of prebiotics

There are many different kinds of food ingredients recognized as prebiotics. Dietary fibers which are composed of carbohydrates (polymers of mono-sugars) are most emphasized and highlighted as prebiotics. Dietary fibers basically resist the hydrolysis by human digestive enzymes in the small intestine; however, they can be fermented by colonic microbiota. Many different kinds of carbohydrates belong to dietary fibers. These include resistant starch (starch and starch degradation products), non-starch polysaccharides (celluloses, hemicelluloses, pectins, gums, and mucilages), inulin, and oligosaccharides such as fructooligosaccharides (FOS, a subgroup of inulin with the degree of polymerization (DP) ≤10), galactooligosaccharides(GOS, DP 2–8), and xylooligosaccharides (XOS, DP 2–10) (Tsai *et al.*, 2019). Other well characterized prebiotics are oligofructose,lactuloseand breast milk oligosaccharides. These ingredients were reported to act thorough enrichment of native *Lactobacillus* spp. and *Bifidobacterium* spp.(Cremon *et al.*, 2018).

## 2.3 Health benefits and mechanisms of action

Several randomized controlled trials have assessed health benefits of orally administered prebiotics on healthy individuals or different target populations, in both acute and chronic diseases. The health endpoints of these studies included functional gastrointestinal disorders (FGIDs), such as irritable bowel syndrome (IBS) and functional constipation; bowel habit and general gut health in infants; traveller's diarrhea; allergy; inflammatory bowel disease (IBD); hepatic encephalopathy; infections and vaccine response; immune function in elderly; necrotizing enterocolitis in preterm infants; urogenital health; skin health; bone health;metabolic health (overweight and obesity; type 2 diabetes mellitus; metabolic syndrome and dyslipidemia). In addition, prebiotics increase the absorption of different minerals, such as iron, calcium, and magnesium, due to their binding/sequestering capacity (Mussatto &Mancilha, 2007) (figure 1).

Prebiotics exert their benefits via the following mechanisms: selective stimulation of the growth and/or activity of intestinal bacteria associated with health, mainly lactobacilli and bifidobacteria (Gibson *et al.,* 2004), and production of short-chain fatty acids (particularly butyrate), which have antimicrobial activity by reduction of intestinal pH and other immunological and physiological activities (Bindels *et al.,* 2015). LowpH values inhibit the growth of certain pathogens, while stimulatethe growth of the bifidobacteria and LAB species (Mussatto &Mancilha, 2007).

## 2.4 Carob as a prebiotic ingredient

The carob tree (*Ceratonia siliqua* L., *Leguminosae* family) is an evergreen tree cultivated or naturally grown in the Mediterranean area such as Greece, Italy, Spain and the Maghreb countries in Nord-Africa including Algeria, Tunisia and Morocco (Rejeb, 1995). The world carob production is approximately 315,000 tons per year, with Spain being the main producer and exporter followed by Italy, Morocco, Portugal, Greece, Turkey and Cyprus. Based on the Food and Agriculture Organisation (FAO) of the United Nations data for the period 1994–2014, Spain produced 74,802.81 tons per year, Italy around 30,000 tons, Morocco and Portugal around 22,000 tons, Greece and Turkey around 15,000 tons, and Cyprus approximately 7000 tons per year (FAO, 2017).

The carob tree is known in Algeria as "Kharroub", "Karrûba", "Taslighoua", "Tikharroubt", "Tikida" (Baba Aissa, 2000). Several Algerian carob varietiesfrom diverse localities were described,indicating a large genetic heterogeneity; type and geographical origin of the trees were taken as the source of diversity (Boublenza *et al.*, 2019). Carob trees can be cultivated in areas with low rainfall, they don't require any significant attention and they live up to 150 years (Hajaji *et al.*, 2011). Due to these characteristics, carobs, have, over the years, been considered as a cheap source for both human and animal nutrition (Ramon-Laca & Mabberley, 2004). Nowadays, carobs are used in the food, pharmaceutical and cosmetic industries (Kotrotsios*et al.*, 2012).

Carob pods (fruits of carob tree) consisting onpulp and seeds, are considered as the main raw material from carob used in industry. The carob pods contain high amounts of carbohydrates (40– 60%), polyphenolic compounds, especially tannins (18–20%), dietary fibers (27–50%), minerals (potassium, sodium, iron, copper, manganese and zinc) and low amounts of proteins (3–

4%) and lipids (0.4–0.8%). This fruit is known especially for its richness in sugars which, are essentially composed of sucrose (32– 38%), fructose (5–7%) and glucose (5–6%), but their relative proportions are variable(Rtibi *et al.,* 2017).

The main categories of phenolic compounds found in carob fruit are phenolic acids, gallotannins and flavonoids. Polyphenols can be found in the carob fruit and more specifically in pulp, seeds and germ. The pulp contains a higher amount of polyphenols in comparison to seeds or germ. The concentration of total polyphenols in carob fruits depends strongly on genetic, environmental and extraction methods and ranges between 45 and 5376 mg gallic acid equivalents per 100 g of dry extract (Stavrou *et al.,* 2018).

The determination of carob's polyphenols revealed the presence of condensed tannins (proanthocyanidins), composed of flavan-3-ol groups and their galloyl esters, gallic acid, (+)-catechin, (-)-epicatechingallate, (-)-epigallocatechingallate, and quercetin glycosides (Corsi *et al.,* 2002; Papagiannopoulos *et al.,* 2004;Ortega *et al.,* 2009) and several authors referred also to the presence of hydrolysable tannins (gallotannins and ellagitannins) in carob pods (Avallone *et al.,* 1997). Owen *et al.* (2003) have identified the polyphenols as tannins, favonoids (26%) and phenolic acids (such as gallic acid, cinnamic acid and p-coumaric acid), favone glycosides (such as quercetin-3-O-a-L-rhamnopyranoside) and hydroxytyrosol. The chemical substances in carob pods differ widely according to carob species, climate and the stage of maturity as well as to different parts of the tree. Indeed, the HPLC analysisshowed that the principal compounds are: pyrogallol (48.02%), catechin (19.10%) and tannic acid (9.01%) in mature carob pods (Rtibi *et al.*, 2015). However, this same technique revealed the abundance ofpyrogallol (26.45%), catechin (16.52%), gallic acid (15.12%), chlorogenic acid (15.01%) and epicatechin (12.26%) in immature carob pods (Rtibi *et al.*, 2016).

It can be noticed that the presence of polyphenols in carob have valuable effect on human health. It is suggested that they can prevent or protect gastric mucosa from acute gastric mucosal injury and promote the healing of chronic gastric ulcers thanks to their antioxidant capacity (Hamaishi*et al.*, 2006). Due to the presence of flavonoids, gallotannins and other associated polyphenols, carob is proposed to be good source of antidiabetic and antioxidant agents (Hasan & Mohieldein, 2016). It has recently been established that the immature carob bean prevents intestinal glucose absorption by the inhibition by electrogenic sodium depended glucose

transport in mice by using a technique of Ussing chamber, which participates in the hypoglycaemic effect (Rtibi*et al.*, 2017).

## 3 Synbiotic: concept, source and health benefits

The additional benefit when prebiotics arecombined with probiotics was speculated by Gibson after he introduced the concept of prebiotic. This combination of prebiotics and probiotics was termed as synbiotics (De Vrese &Schrezenmeir, 2008). Thus "synbiotic" beneficially affects the host by improving the survival and selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria in the GI tract. The products in which the prebiotic compound(s) selectively favour the probiotic organism(s) are true synbiotics (Cencic &Chingwaru, 2010). It also helps to overcome the possible survival difficulties for probiotics. Fermented milk is considered a synbiotic as it provides both live beneficial bacteria (probiotics) and products of fermentation that may affect the intestinal microflora in a positive way (prebiotics) (Famularo *et al.,* 1997).

For synbiotics formulation, the probiotic strains used include *Lactobacillus*, *Bifidobacterium* spp., *Saccharomyces boulardii, Bacillus coagulans*, etc., while the major prebiotics used comprise oligosaccharides like fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and xylose-oligosaccharides (XOS), inulin, prebiotics from natural sources like chicory and yacon roots (Mishra & Mishra, 2018).

The main health benefits of synbiotics are **(1)** increase in the balance of gut microbiota, **(2)** improvement of liver function in cirrhotic patients, **(3)** improvement of immune-modulating ability,and **(4)** prevention of bacterial translocation and reduction of nosocomial infections incidences in surgical patients, etc. (Zhang *et al.* 2010). Indeed, clinical evidences for efficacy of synbiotics use in treatment of a number of human digestive disorders were presented recently (figure 2).

The most common additives to probiotic strains are usually inulin and FOS. Supplemented with inulin, the following strains were used for successive treatment of the respective diseases: *Bf. lactis* B94 against infectious diarrhea in children (Islek *et al.,* 2014); *Bf. longum* (Synergy 1) against Crohn's disease (Steed *et al.*, 2010); Prebiotic–Probiotic Relationship 267 *Lb. sporogenes* against diabetes (Asemi *et al.,* 2014). Mix of several probiotic

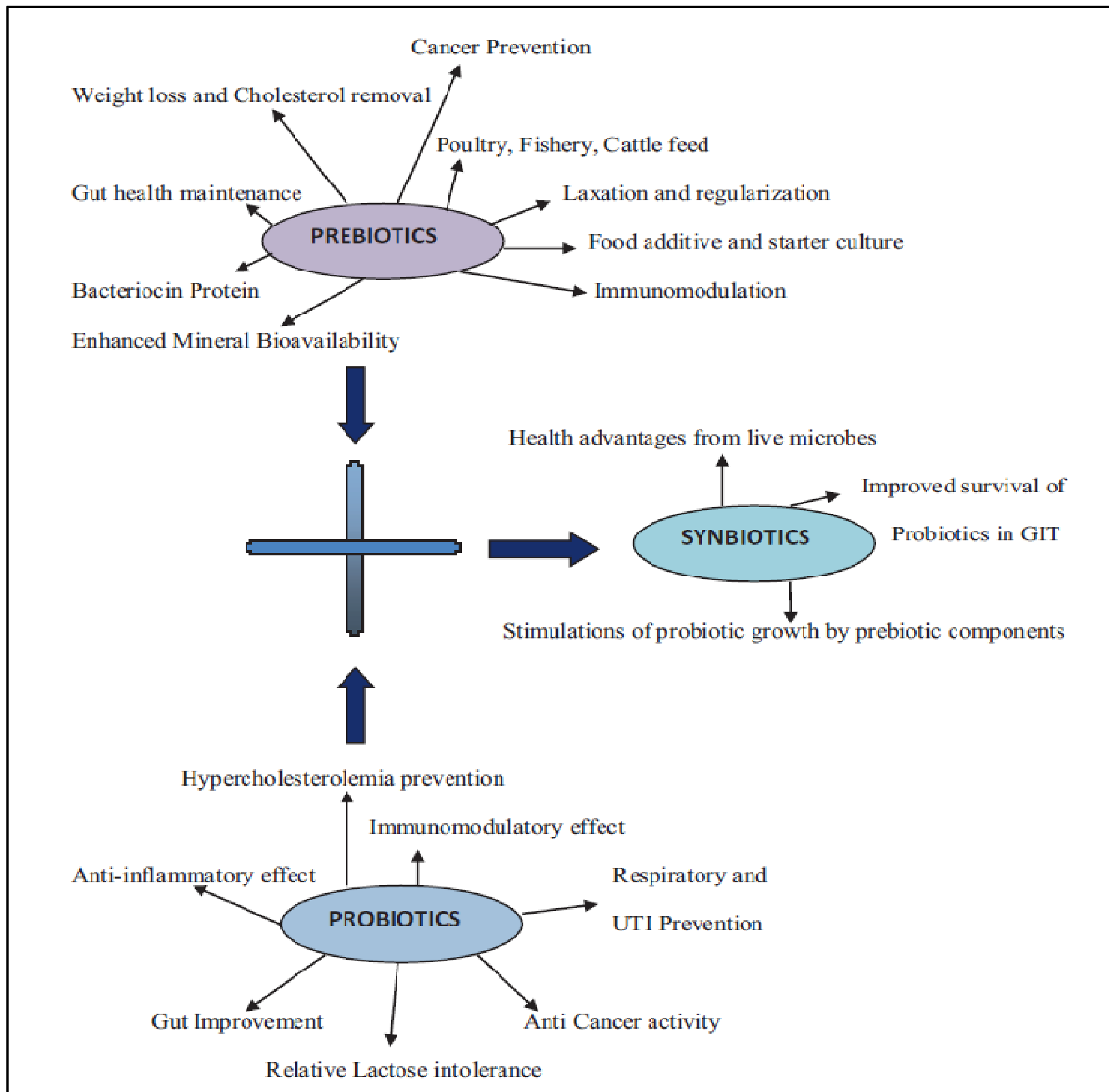


**Fig. 2:** Pictorial summary of prebiotics, probiotics and synbiotics and their uses (adapted from Mishra & Mishra, 2018). GIT: GastroIntestinal Tract, UTI: Urinary Tract Infections.

strains (*Lb. paracasei* Lpc-37, *Lb. rhamnosus* HN001, *Lb. acidophilus* NCFM, and *Bf. lactis* HN019), and 6 g of FOS per sachet were used in Lactofos formula for treatment of constipation (Waitzberg *et al.*, 2013). *Lb. casei, Lb. rhamnosus, Str. thermophilus, Bf. breve, Lb. acidophilus, Bf. longum,* and *Lb. bulgaricus* in combination with FOS (Protexin) alleviate the symptoms of nonalcoholic fatty liver disease (Eslamparast *et al.,* 2014). Inulin (up to 20%) was used for

development of an encapsulated oral-synbiotic containing three probiotic strains: *Pediococcus acidilactici, L. reuteri,* and *L. salivarius* (Atia *et al.,* 2015).

# 4 Fermented milks

## 4.1 Fermented milks and Definition

Fermentation has long been used as a way to naturally enhance the food matrix, without the need for additives or preservatives (Hugenholtz, 2013). The fermentation of foods involves the breaking down of complex constituents into simpler ones, many of which can possess bioactive properties, through the use of microorganisms. Nutritional and functional properties are enhanced through the fermentation process;these include the improved availability of trace minerals, vitamins and antioxidants as well as greater safety, shelf-life and sensory attributes (Macori &Cotter, 2018).

Fermented milks represent a major portion of the total existing fermented dairy products.Theyare generally defined as those beverages made through controlled microbial growth and enzymatic conversions of major and minor milk components (Marco *et al.*, 2017). Fermented milk is produced through the coagulation of milk, without the elimination of serum, by bacterial cultures that generally remain present until consumption (Marco *et al.*, 2017).

Adapting a classification scheme proposed by Robinson &Tamime (1996), which takes into account the kind of microorganisms dominating the fermentation and the majority sensory metabolites of the fermented products, two fundamentally different fermented milk classes can be proposed (Shiby &Mishra, 2013):

***Class A***: lactic fermentations, in which LABlead the fermentation changes. The products, within this group,constitue the largest consumed ones worldwide.They can be subdivided into three subclasses depending on the microbial types depending on fermentation pattern: ***Subclass Ai***: mesophilic type; for example, natural acidified milk, cultured milk, cultured cream, cultured buttermilk, filmjölk, and långfil. ***Subclass Aii***: thermophilic type; for example, yogurt, Bulgarian buttermilk, zabadi, and dahi. ***Subclass Aiii:*** probiotic/therapeutic type; for example, acidophilus milk, yakult, and bifidus milk.

***Class B***: Yeast lactic fermentations, where LAB and yeasts species cooperate to generate the final product. These fermentations can be further separated into two subclasses: ***Subclass Bi:*** alcoholic milks; for example, kefir, koumiss, and acidophilus yeast milk. ***Subclass Bii:*** moldy milks; for example, viili.

### 4.2 Health benefits of fermented milks

The health benefits of dairy products,includingfermented milks, are the result of biologically active components that are present in native milk and also, owing to their suitably modulated activities, produced through the action of probiotic bacteria in fermented or sour milk products. In addition to modification of several milk components, the probiotics may also act directly as preventive or curatrive agents of some contagious, atopic, tumorous, or other severe diseases (Gill &Guarner, 2004; Santosa *et al.,* 2006). The health-promoting effects of the fermented functional foodstuffs materialize directly through interactions with consumed microorganisms (probiotic effect) or, indirectly, as a result of the action of microbial metabolites generated during the fermentation process (biogenic effect). The most important biogenic metabolites include vitamins, proteins, peptides, oligosaccharides, and organic acids, including fatty acids.

Moreover, human clinical studies on fermented milks revealed that mechanism of probiotics action is based on the positive effect they exert on the immune response, i.e., on the immunomodulatory activity (Biancone *et al.,*2002). In addition, large cohort investigations have revealed strong associations between consumption of fermented dairy foods and weight maintenance (Mozaffarian, 2011). Likewise, other long-term prospective studies show reductions in risk of cardiovascular diseases (CVD), type 2 diabetes (T2D), and overall mortality from frequent yogurt consumption (Chen *et al.,* 2014; Eussen *et al.*, 2016).

### 4.3 Traditional fermented milks in Algeria

Contrary to popular belief, Algeria does have well established traditions of manufacturing dairy products even if the activity is limited to the domestic sphere. The traditional dairy products, with strong cultural, medicinal, and economic value, are the historical product of the social and economic dynamism of the rural communities of women (Claps &Morone, 2011).

Despite their anchoring in the Algerian culinary tradition and their ability to exploit the natural resources of disadvantaged regions (animal and plant species of mountain and Saharanecosystems), these products evolved on the margins of implemented development policies in Algeria. The great majority of them are being downgraded by the markets through the emergence of dairy processing industries oriented more towards the satisfaction of the major urban markets, in subsidized milk and dairy products, than towards the valuation of local milk production (McSweeney *et al.,* 2017). Many traditional products are vanishing for various reasons, including lack of feed availability, rural exodus, and changing dietary habits. Those ones whose; use is most widespread, such as Raib and Lben, and while keeping the same name, have changed their technological process because of their industrialization (Benkerroum, 2013).

**"Raib"**: is fermented milk produced in many Mediterranean and sub-Saharan countries (Licitra *et al.*, 2019). "Raib" is curdled milk, traditionally obtained after spontaneous acidification at room temperature of raw milk during a period ranging from 24 to 72 h; it is consumed as it is or transformed. Fermentation has been associated with mesophilic LAB belonging to the genera *Leuconostoc* and *Lactococcus* naturally present in raw milks (Mechai *et al.,* 2014).

**"Lben"**: is one of the best-known products of artisanal milk processing; it is prepared following the churning of "Raib" and butter separation (Jans *et al.,* 2107). Churning makes it possible to collect the majority of its fat in the form of butter called "Zebda." The homemade or family preparation of "Lben" is simple: the milk is left to itself in a "Rawaba" terracotta pot until it coagulates. This is done at room temperature and lasts 24 to 72 h (Leksir *et al.,* 2018).

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**Part 2: Experimental study**

## Chapter 1: Isolation, screening and characterization of lactic acid bacteria for their starter and probiotic potentialities from Algerian artisanal cheeses

This chapter corresponds to the first objective of this thesis which aims to isolate new lactococci and lactobacilli strains with specific properties to be used as Starters and Probiotics respectively.

The lactoccoci isolates were studied for their antimicrobial activity and technological properties in a purpose to choose the most performant one to be used as a starter for the formulation of the carob fermented milk. However, the lactobacilli isolates were selected based on their antimicrobial, technological as well as their probiotic properties to be used as probiotic strain in the carob fermented milk.

A part of this chapter was published in Folia Microbiologica journal.

### 1 Material and methods

#### 1.1 Samples collection and isolation of *Lactococcus* and *Lactobacillus* strains

Five (5) fresh, soft, artisanal homemade cheeses were collected from five different areasin Bejaia city (North East of Algeria) for the sake of isolating of lactococci and lactobacilli strains. Ten grams (10 g) of each sample were homogenized in 90 mL of 2 % (w/v) sterile warm sodium citrate solution for 5 min. Serial decimal dilutions was performed and appropriate dilutions were inoculatedin acidified de Man, Rogosa and Sharpe (MRS) agar (pH = 5.4) for lactobacilli or in M17 for lactococci (Merk, Germany) isolation. Plates were then incubated aerobically at 30°C for 72 h(Harrigan,1998). Five randomly selected colonies were purified by subsequent culturing and were preliminarily identified based on their morphological and staining characteristics (Gram-positive cocci and bacilli) and negative catalase reaction (3 % [v/v] $H_2O_2$). Pure cultures were stored at -20°C in MRS/M17 broth supplemented with 20 % (v/v)sterile glycerol.

#### 1.2 Screening of lactococci and lactobacilli strainsfor their antibacterial activity

The inhibitory effect of lactococci and lactobacilli strains was detected via the spot-on-lawn method and well-diffusion assay according to Bendali*et al.*(2011) using *Escherichia coli*

ATCC 25922,*Staphylococcus aureus* ATCC 25923, *Salmonella enterica* TyphimuriumLT2 and*Listeria innocua* CLIP 74915as target strains. Briefly, 5 µL of 18- h bacterial cultures were spotted onto MRS/M17 agar plates and incubated aerobically at 30°C for 18 h. The spots were then overlaid with a soft Brain Heart Infusion (BHI) agar (0.75% [w/v] agar) seeded with $10^6$ CFU /mL of the target strain before being incubated at 37°C for 18 h. Lactococci/lactobacilli strainsshowing inhibition zones surrounding the bacterial spots were further studied with the well-diffusion assay to identify the inhibitory substances secreted into the growth medium. For this, the cell-free supernatants (CFSs) of 18-h cultures, obtained by centrifugation at 8000 g/20 min at 4°C(Thermo scientific, Sorval legendXTR, Germany),were filter sterilizedusing0.22 µm filters (Celltreat scientific product, China) and their pH was measured (HANNA instruments HI 2211, Italy). Then, the CFSs were divided into four samples, sample 1 was retained as an untreated sample, sample 2 was adjusted topH6.5 to determine the organic acid function, sample 3 was heated to 100°C for 15 min to test heat sensitivity and sample 4 was incubated with 1 mg/ mL of proteinase K at 37°C for 3 h to assess the effects of the proteases. Prepared MRS agar plates were overlaid with 10 mL of soft BHI agar mixed withthe targetstrain ($10^6$ UFC/mL). Wells (6 mm diameter) were punched in the agar layer and filled with 100 µL of treated or untreated CFSs. The plates were incubatedaerobicallyat 37°C for 24 h. The diameters of the inhibition zones were measured, and halos of more than 3 mm were considered positive.

### 1.3 Technological characterization of lactococci and lactobacilli strains

Lactococci and lactobacillicells, grown in MRS and M17 broths respectively at 30°C for 18 h, were harvested by centrifugation (8000g, 20 min, 4°C), washed twice, re-suspended in PBS (pH=7.5) and subjected to the following tests.

#### 1.3.1 Heat resistance

A heat tolerance assay of lactococci or lactobacilli strainswas performed by the method described by Paéz *et al.* (2012). Briefly, cells were re-suspended in 10% (w/v) skimmed milk and placed in a water bath at 60°C for 5 min. The suspensions were then immediately cooled in an ice bath and the viable cells were counted before and after exposure to heat.

### 1.3.2 Acidifying activity

Acidifying capacity was evaluated by inoculation ofthe lactococci or lactobacilli strains ($10^5$ CFU/ mL) in 10 mL of sterile reconstituted skimmed milk (10%, w/v). The suspensionswere then incubated at 30°C. After 18 h of incubation, samples were taken for cell growth and pH measurements (Zhaoxu*et al.*, 2018).

### 1.3.3 Proteolytic activity

The proteolytic activity of the lactococci and lactobacilli strains was determined via qualitative assays as previously described by Guiraud (1998). An aliquot (5 µL) of a fresh culture was spotted on MRS/M17 agar plates supplemented with 10% (w/v) sterile reconstituted skimmed milk. The plates were then incubated at 30°C for 48 h. A precipitation zone surrounding the spots was taken as a positive indicator of proteolysis.

## 1.4 Probiotic properties of lactobacilli strains

### 1.4.1 Resistance to simulated gastrointestinal digestion

Slightly modified, the method reported by Saito *et al.* (2014) was adopted in order to assess the survivability of the *Lactobacillus* strains under gastrointestinal conditions. Cultures (30°C/18 h) of lactobacilli strains were centrifuged (8000 g, 20 min, 4°C), the pellets werewashed twice with Phosphate Buffered Saline (PBS, pH=7.2, 10mM) and re-suspended in 10 mL of sterile reconstituted skimmed milk (10 % [w/v] in sterile water). This suspension was then mixed with the same volume of a simulated saliva-gastric solution containing $CaCl_2$ (0.22 g/L), NaCl (16.2 g/L), KCl (2.2 g/L), $NaHCO_3$ (1.2 g/L), and 0.3 % (w/v) pepsin (Sigma, Canada). One milliliter samples were removed immediately after mixture in order to count the lactobacilli cell numbers (before pH adjustment at 2.5 with 6M HCl) and after 60 and 120 min of incubation at 37°C in a water bath. The remainingcultureswere centrifuged (8000 g, 20 min, 4°C) and re-suspended to the original volume in MRS broth containing 1% (w/v) bile salts (Sigma, Canada) at pH 7.5 (bile shock). Cell viability was assessedbefore and after incubation at 37°C for 10 min. Similarly, cultures were centrifuged, and the pellets were re-suspended to the original volume in MRS broth containing 0.3 % (w/v) bile salt (Sigma, Canada) plus 0.1 % (w/v) pancreatin (Sigma, Canada) at pH 7.5. One millilitre was taken before and after an incubation period of 180 min at 37°C to assess cell viability.

### 1.4.2 Determination of lactobacilli strains adhesion ability

The adhesion ability of the strains was estimated as a measure of their hydrophobicity, autoaggregation and co-aggregation.

**Cell surface hydrophobicity**

The cell surface hydrophobicity (H%) was determined by bacterial adhesion to an apolar hydrocarbon (xylene) according to Kos *et al.* (2003) with some modifications.Overnight *Lactobacillus* cultures in MRS broth were centrifuged for 20 min at 8000 g at 4°C, washed twice and resuspended in PBS. Three milliliters of these suspensions were added to 0.6 mL of xylene(Sigma) and vortexed for 2 min. The two phases were allowed to separate for one hour at 37°C. The aqueous phase was carefully removed and the OD600 nm was measured.

The cell surface hydrophobicity (H%) was calculated as follows:

$$\text{H\%} = 1 - \frac{A1}{A0} * 100$$

Where A0 and A1 are respectively the absorbenciesbefore and after the addition of xylene.

**Autoaggregation**

The autoaggregation (Auto%) ability of the lactobacilli strains was assessedas described by Kos *et al*. (2003). Overnight lactobacilli cultures in MRS broth were centrifuged for 20 min at 8000 g at 4°C, washed twice and resuspended in phosphate buffered saline (PBS, 3 mL), vortexed for 30 s and incubated for 2 h at 37°C. An aliquot (1 mL) of these suspensions was carefully removed from the upper zone, and the OD600nm was measured before and after incubation.

The autoaggregation (Auto%) was expressed as follows:

$$\text{Auto\%} = 1 - \frac{At}{A0} * 100$$

Where At represents the absorbance at t= 5 h, and A0 the absorbance at t=0.

**Co-aggregation**

The co-aggregation potential was performed according to slight modified method toSolieri*et al.* (2014) using *Escherichia coli* ATCC 25922 or*Staphylococcus aureus* ATCC

25923 as the mixed strain.The lactobacilli and pathogens strains were separately cultured at 37°C for 18 h in MRS and TSB medium. Bacterial suspension ($10^8$CFU/ ml) were formulated as described in the auto-aggregation in above method, equal volume of cells of the different *Lactobacillus*strains and pathogenic strains (1:1 v/v) were mixed and incubated at 37°C without agitation. Absorbance at 600 nm of the mixture represent above, was supervised during incubation at 4 h.

Percentage of co-aggregation were directed as:

$$\text{Coaggregation (\%)} = [(A_{pathogen} + A_{Lactobacillus})] / 2 - \text{Amix } (A_{pathogen} + A_{Lactobacillus}) / 2 * 100$$

Where, $A_{pathogen}$ and $A_{Lactobacillus}$ and $A_{mix}$ represent the A600 of individual pathogen, *Lactobacillus* spp. and their mixture after incubation for 4 h, respectfully.

### 1.4.3 Adhesion to intestinal HT-29 cells

The adhesion ability of the lactobacilli strains was studied using HT-29 cells as previously described by Waśko*et al.* (2014) with some modifications. Cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10 % (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) mixture of penicillin-streptomycin solution, at 37°C in an atmosphere of 5% $CO_2$ (Thermo Scientific, water jacketed$CO_2$, Germany). Cells were then seeded onto a 6-well tissue culture plate at a density of 4 x $10^4$ cells/mL. After 24 h of incubation, overnight bacterial cultures ($10^8$ CFU/mL) re-suspended in DMEM medium without serum and antibiotics, were added to the monolayer of HT-29 cells. The monolayers were washed twice with sterile PBS after 2 h of incubation to remove the non-adherent bacteria and then lysed using 0.1% (v/v) Triton-X100. The lysates were serially diluted and plated onto MRS agar to enumerate the number of lactobacilli cells that had adheredto HT-29. The percentage of bacterial adhesion was calculated as follows:

$$\text{bacterial adhesion (\%)} = \frac{adhered\ bactreria}{total\ added\ bacteria} * 100$$

### 1.4.4 Antibiotic susceptibility

The antibiotic resistance profile of lactobacilli strains was investigated by the agar diffusion method (Charteris *et al.*,1998). The strains were grown on MRS agar in aerobiosis (30° C, 18 h). Colonies were transferred to tubes containing 3.5 ml of saline solution (0.85% w/v

NaCl) and adjusted to 0.5 McFarland turbidity. Lactobacilli were inoculated on MRS agar and disks (Oxoid, Basingstoke, England) containing the different antibiotics were distributed on the agar surface.The following antimicrobial agents weretested: penicillin G (10 µg) and vancomycin (30 µg) as inhibitors of cell wall synthesis; kanamycin (30 µg), chloramphenicol (30 µg), tetracycline (30 µg), streptomycin (10 µg) as inhibitors of protein synthesis and rifampicin (10 µg) as aninhibitor of nucleic acid synthesis. The diameter of the inhibition zones was measured and compared with the breakpoint values reported by Charteris*et al.* (1998), to classify strains as resistant (R), moderately sensitive (MS), orsensitive (S).

### 1.4.5 Cholesterol lowering potential

The ability of lactobacilli strains to remove cholesterol was evaluated as described by Lavanya *et al.* (2011) by cultivating them in 100 mL of MRS broth supplemented with 0.5% (w/v) bile salts (Sigma, Canada) and filter-sterilized cholesterol solution (10 mg dissolved in 500 µl of ethanol) (Sigma, Canada). Non-inoculated broth was considered as a control. The concentration of cholesterol was determined using a cholesterol standard curve. The percentage of cholesterol assimilation was determined using the formula:

$$\text{Assimilation } \% = \frac{[Cholesterol\ in\ control] - [Cholesterol\ in\ sample] * 100}{[Cholesterol\ in\ control]}$$

### 1.4.6 Antioxidant activity of *Lactobacillus* strains

Antioxidant activity was examined by the DPPH (1-diphenyl-2-picrylhydrazyl) scavenging activity of theCFS of lactobacilli culturesaccording to Das & Goyal (2015). The 100 µl of ethanolic DPPH solution (0.4 mmol/L) was mixed vigorously with 100 ml of intact cells ($10^9$ CFU/ml) or water (control) and incubated at 37°C in the dark for 30 min. The absorbance (A) was measured at 517 nm against a blank of DPPH solution (control) and the scavenging ability was calculated according to the following equation:

$$\text{Scavenging ability } (\%) = 1 - \left(\frac{A\ of\ supernant}{A\ of\ control}\right) * \ 100$$

### 1.5 Molecular identification of the selected strains

#### MALDI-TOF spectrometry

The identification of selectedlactobacilli strains by Maldi-Tof spectrometry was performed as described by Ait Seddik*et al*. (2017).

#### 16S rDNA sequencing

The 16S rDNA sequence analysis was done at McGill University and Génome Québec Innovation Centre by the Sanger sequencing (Montréal, QC, Canada). Total DNA was amplified by PCR using the universal 27 forward primer:

(5'ACACTGACGACATGGTTCTACAAGAGTTTGATCCTGGCTCAG 3') and the universal 1492 reverse primer:

(3'TACGGTAGCAGAGACTTGGTCTGGTTACCTTGTTACGACTT 5').

The following program was used: 94°C for 2 min, 35 cycles of 94°C for 45 s, 52°C for 60s, 72°C for 60s, and finally 72°C for 7 min. The obtained sequence was aligned with NCBI using BLAST database.

### 1.6 Statistical analyses

Statistical analyses were performed using IBM's SPSS Statistic software, version 24. The data are expressed as mean ± standard deviation of triplicate experiments. Significant one way-ANOVA results were followed up with Tukey's post-hoc test in all assays and differences were considered statically significant when $p < 0.05$.

## 2 Results and discussion

### 2.1 Screening of lactococci and lactobacilli strainsfor their antibacterial activity

A total of 118bacterial strains belonging to *Lactococcus*(C1 to C20) and *Lactobacillus*(B1- B98) genera were isolated from soft Algerian artisanal cheeses. As antibacterialactivity is one of the crucial properties used to select starters and probiotics, all the strains were screened for their ability to produce antibacterial agents against food-borne pathogens (*S. aureus*,*E. coli, Salmonella enterica*and *Listeria monocytogenes*).According to their

morphological and cultural characteristics (Gram, Catalase,growth at 45°C, growth in 6,5% salt, growth in pH 9.2, …), 20isolates seemed to be presumptive lactococci. In agar spotmethod, *S. aureus*,*E. coli*, *Salmonella* and *L. monocytogenes* were suppressed by 16 lactococci isolates with inhibition zones diameters varing from14 to 34 mm. The evaluation of the bacteriocin-like inhibitory substances with the well diffusion technique revealed that the native CFS (pH = 4.35 – 4.89) of 10 isolatesshowed an antibacterial effect against all indicator strainswith inhibition zones diameters going from 9 to 17 mm (Table 2; Table 1 in appendix). Only one isolate (C15) was able to inhibit the growth of *S. aureus*when the CFS was neutralized with an inhbition zone diameter of 15 mm; however no inhibitory activity was detected with the same strain against other strains. In addition, the antibacterial effect of C15 against *S. aureus* remaind active after proteinase K and heat treatments, which suggest that this activity may be due to a production of a bacteriocin- like substance.

In another hand, a total of 98 isolates wereassumed to belong to *Lactobacillus* genus.Only 65 strains had exhibited an inhibitory activity with the spot-on-lawn method against both pathogens (Table 1). Out of these lactobacilli strains, only 14 expressed antibacterial activityusing the native CFS (pH = 3.69 - 4.23), when tested with the well diffusion method (Table 2). Those 14 lactobacilli strains exhibited potent antibacterial activity against both *E. coli* ATCC25922 and *S. aureus* ATCC 25923. Comparatively, the 14 strains showed greater antagonism ($p < 0.05$) towards *E. coli* (8-15 mm) than towards *S. aureus* (5-10 mm). Among these strains, only three strains (B9, B13 and B38) were anatgonistics to *Salmonella enterica* (9, 12 and 10 mm) and *L. monocytogenes* (10, 12 and 9 mm) (Appendix Table 1). These results are in agreement with a previous study reported by Riaz Rajoka *et al.* (2017) with lactobacilli strains isolated from human milk.

However, this antibacterial activity disappeared when the CFS was neutralized to pH 6.5 and remained active after proteinase K and heat treatments (Table 2). This suggests that the involvement of bacteriocins in the inhibitory activity could be discarded as an explanation and that the antibacterialactivity of the strains may be attributed solely to the effects of organic acids.

**Table 1**. Antimicrobial activity of lactobacilli strains against indicator strains with the spot-on-

| Diameter of inhibition zones (mm) | *E. coli* ATCC 25922 | *S. aureus* ATCC 25923 |
|---|---|---|
| 0 | 33 | 33 |
| < 10 | 3 | 16 |
| 10 – 20 | 15 | 17 |
| 20 -30 | 25 | 27 |
| > 30 | 22 | 5 |

lawn method

It is frequently documented that organic acids (mainly lactic acid) exhibit strong antimicrobial activity against different pathogens such *E. coli*, *Pseudomonas aeruginosa* and *Salmonella enteric*a (Alakomi *et al.*, 2000). The mechanism of action of organic acids has been reported as being due to their undissociated form which diffuses into microbial cells and decreases the pH of cytoplasm by dissociating within, becoming inhibitory to microorganisms (Chaveerach, 2002; Sundberg & Jönsson, 2005). Wang *et al.* (2014) reported that the CFSs of lactobacilli isolated from traditional koumiss expressed higher antibacterial activity against *Compylobacter jejuni* than those isolated from healthy infant feces. These explain the relationship between the organic acids production ability and the antimicrobial activity of the strains isolated from fermented products (Wang *et al*., 2014).

The 14 lactobacilli strains selected based onthis characteristic were further investigated using the following tests summarized below.

**Table 2.** Antimicrobial activity of cell free supernatants (CFSs) of lactococci / lactobacilli strains against indicator strains (mm)

| Strains | Inhibition zone (mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CFSs | | | Neutralized CFSs (pH=6.5) | | Heat (100°C, 15 min) | | Proteinase K | |
| | pH | *E. coli* | *S. aureus* | *E. coli* | *S. aureus* | *E. coli* | *S. aureus* | *E. coli* | *S. aureus* |
| C15 | 3.18 | 17± 1.02$^{bc}$ | 15± 0.44$^{bc}$ | 0 | 15± 0.54$^{cde}$ | 0 | 14± 0.54$^{cde}$ | 0 | 15± 1.02$^{cd}$ |
| B1 | 3.69$^{a}$ | 15 ± 0.66$^{e}$ | 8 ± 0.52$^{cde}$ | 0 | 0 | 14 ± 0.86$^{e}$ | 8 ± 0.21$^{cde}$ | 14 ± 0.24$^{e}$ | 8 ± 0.44$^{cde}$ |
| B3 | 4.23$^{b}$ | 11 ± 0.45$^{b}$ | 8 ± 0.47$^{cde}$ | 0 | 0 | 11 ± 0.93$^{b}$ | 8 ± 1.24$^{cde}$ | 10 ± 0.68$^{b}$ | 8 ± 0.38$^{cde}$ |
| B5 | 4.20$^{b}$ | 13 ±0.33$^{bcd}$ | 6 ± 0.24$^{ab}$ | 0 | 0 | 12 ± 0.41$^{bcd}$ | 5 ± 1.36$^{ab}$ | 12 ±0.75$^{bcd}$ | 6 ± 0.87$^{ab}$ |
| B9 | 3.80$^{cd}$ | 13 ± 0.98$^{cde}$ | 9 ± 1.14$^{def}$ | 0 | 0 | 13 ± 0.22$^{cde}$ | 9 ± 0.32$^{def}$ | 13 ±1.32$^{cde}$ | 9 ± 1.25$^{def}$ |
| B13 | 3.83$^{d}$ | 11 ± 1.02$^{bc}$ | 10 ± 0.35$^{f}$ | 0 | 0 | 10 ± 1.14$^{bc}$ | 10 ± 0.45$^{f}$ | 11 ± 0.99$^{bc}$ | 10 ± 0.99$^{f}$ |
| B16 | 4.03$^{e}$ | 10 ± 0.47$^{b}$ | 10 ± 0.12$^{f}$ | 0 | 0 | 10 ± 0.35$^{b}$ | 9 ± 0.66$^{f}$ | 10 ± 0.67$^{b}$ | 9 ± 0.23$^{f}$ |
| B18 | 3.82$^{d}$ | 15 ± 0.53$^{e}$ | 8 ± 0.93$^{cd}$ | 0 | 0 | 15 ± 0.44$^{e}$ | 8 ± 0.47$^{cd}$ | 14 ± 0.35$^{e}$ | 9 ± 0.47$^{cd}$ |
| B19 | 3.74$^{c}$ | 14 ± 0.66$^{de}$ | 8 ± 0.56$^{cd}$ | 0 | 0 | 14 ± 0.75$^{de}$ | 8 ± 0.41$^{cd}$ | 13 ± 0.49$^{de}$ | 7 ± 0.94$^{cd}$ |
| B20 | 3.85$^{d}$ | 13 ± 0.21$^{cde}$ | 7 ± 0.44$^{bc}$ | 0 | 0 | 13 ± 0.54$^{cde}$ | 7 ± 0.22$^{bc}$ | 12 ±0.25$^{cde}$ | 7 ± 0.25$^{bc}$ |
| B21 | 3.76$^{cd}$ | 13 ±0.74$^{bcd}$ | 8 ± 0.74$^{cd}$ | 0 | 0 | 13 ± 0.42$^{bcd}$ | 9 ± 1.23$^{cd}$ | 13 ± 0.16$^{cd}$ | 8 ± 1.02$^{cd}$ |
| B27 | 3.84$^{d}$ | 11 ± 1.03$^{b}$ | 9 ± 1.52$^{ef}$ | 0 | 0 | 12 ± 0.74$^{b}$ | 9 ± 0.66$^{ef}$ | 12 ± 1.33$^{b}$ | 9 ± 0.57$^{ef}$ |
| B34 | 3.98$^{f}$ | 10 ± 0.61$^{bc}$ | 5 ± 0.84$^{a}$ | 0 | 0 | 10 ± 0.21$^{bc}$ | 5 ± 0.85$^{a}$ | 10 ± 1.02$^{bc}$ | 4 ± 0.66$^{a}$ |
| B35 | 4.23$^{b}$ | 10 ± 0.74$^{b}$ | 10 ± 0.61$^{ef}$ | 0 | 0 | 9 ± 0.36$^{b}$ | 10 ± 0.74$^{ef}$ | 10 ± 0.99$^{b}$ | 10 ± 0.65$^{ef}$ |
| B38 | 3.83$^{d}$ | 8 ± 0.26$^{a}$ | 9 ± 0.22$^{def}$ | 0 | 0 | 8 ± 0.54$^{a}$ | 8 ± 0.68$^{def}$ | 9 ± 0.66$^{a}$ | 8 ± 0.23$^{def}$ |

## 2.2 Technological properties

The technological properties as the acidification activity areimportant characteristics for the selection of LAB strains to be used as primary or secondary cultures in dairy fermentation. Heat resistant and fast acid producing strains are commonly used as starter cultures whereas poor acid producers can be used as adjunct cultures (Ayad *et al.*, 2004).According to their acidification activity, the lactococci isolates were grouped as fast, medium or slow acidifiers. A 45% of the strains were considered as fast acidifiers reducing the pH of milk from 7.56 to 5.3 in less than 6 h at 30°C (Cogan *et al.*, 1997).These results are in agreement with the study conducted by (Ho *et al.,* 2018).

Resistance to heat is an important factor for the industrial application of a starter or a probiotic since it must maintain its viability during the food manufacturing process and throughout its shelf-life and until consumption(Pino *et al.*, 2017). Most of the tested lactococci strains exhibited an interesting heat resistance with a reduction in viable cells number less than one logarithm. In addition, the lactococci isolates showed a high proteolytic activity with precipitated zones ranged from 25 to 34 mm.LAB demonstrate variable amounts of the proteases and peptidases involvedinhydrolyzingmilk caseininto smaller peptides and the free amino acids which can contribute to the formation of flavour and texture in dairy products (Settanni & Moschetti, 2010). Previous studies demonstrated that proteolysis lead to the generation of bioactive peptides with immunomodulatory, antihypertensive, antioxidative, antimicrobial and other health-promoting properties (Hayes*et al.*, 2007; Wakai & Yamamoto, 2012).

According to the obtained results from the antibacterial activity as well as the technological properties, the isolate *Lactococcus* C15 showed interesting characteristicsand was chosen for molecular identification and fermented milk production.

The technological properties of the 14 selected lactobacilli strains tested in this study are illustrated in table 3. After exposing the lactobacilli strains to a heat treatment of60 °C for 5 min in 10 % (w/v) skim milk, the reductions inviablecells counts ranged from 0.95 to 3.59 logs units.StrainB19 showed the highest resistanceto this treatment. These results are in agreement with those of (Teles Santos *et al*., 2016).

The acidifying ability of lactobacillistrainswas also tested in 10% (w/v) skim milk. Most of the strains showed a low acid production after 18 h of incubation (pH between 5.42 and 7.14) compared to the initial pH of skim milk (pH=7.56). Only strainsB16 and B19 exhibited high acidification with pH values of 4.02 and 4.78 respectively. These results may be due to theslowness with which the*Lactobacillus* genus metabolizes lactose (González*et al.*, 2010; Herreros*et al.*, 2003).

Concerning their proteolytic activity, the tested strains of lactococci as well as lactobacilli demonstrated sizeable precipitated zones ranging from 21 mm to 30 mm. These results are in contrast with the study of (Carafa *et al.,* 2015) who report no exoproteolytic activity in lactobacilli isolated from Mountain Cheese.The testedlactobacillistrains produced proteolytic enzymes which could be useful in fermented food products.

**Table 3.** Technological properties tested for lactobacilli / lactococci strains

| Strains | heat resistance | | Acidifying activity | | Proteolytic activity |
|---|---|---|---|---|---|
| | 0 min | 5 min | pH | Number of cells | |
| C15 | $9.34 \pm 0.36^{i}$ | $9.04 \pm 0.66^{g}$ | $3.45 \pm 0.47^{abd}$ | $9.12 \pm 0.36^{g}$ | $30 \pm 0.74^{ad}$ |
| B1 | $8.64 \pm 0.65^{a}$ | $6.39 \pm 0.25^{a}$ | $6.12 \pm 0.22^{abce}$ | $7.83 \pm 1.11^{a}$ | $21 \pm 0.44^{ad}$ |
| B3 | $8.77 \pm 0.99^{bg}$ | $5.18 \pm 0.55^{b}$ | $7.03 \pm 0.65^{bd}$ | $6.86 \pm 0.33^{b}$ | $26 \pm 0.66^{bf}$ |
| B5 | $8.92 \pm 0.45^{c}$ | $6.51 \pm 0.84^{c}$ | $5.42 \pm 0.47^{ef}$ | $7.15 \pm 0.99^{c}$ | $23 \pm 1.14^{ac}$ |
| B9 | $8.67 \pm 0.84^{ad}$ | $7.46 \pm 1.11^{d}$ | $6.27 \pm 0.33^{abcde}$ | $7.51 \pm 0.54^{de}$ | $22 \pm 0.74^{ad}$ |
| B13 | $8.11 \pm 0.32^{e}$ | $7.61 \pm 0.35^{e}$ | $5.71 \pm 0.14^{acef}$ | $7.40 \pm 0.78^{ei}$ | $27 \pm 0.47^{bf}$ |
| B16 | $8.56 \pm 0.14^{f}$ | $7.17 \pm 0.96^{f}$ | $4.02 \pm 0.65^{g}$ | $8.36 \pm 0.54^{f}$ | $25 \pm 0.65^{cf}$ |
| B18 | $8.95 \pm 1.02^{c}$ | $6.20 \pm 0.44^{g}$ | $5.66 \pm 047^{cef}$ | $7.69 \pm 0.36^{g}$ | $30 \pm 0.94^{e}$ |
| B19 | $8.80 \pm 1.11^{g}$ | $7.85 \pm 0.63^{i}$ | $4.78 \pm 0.36^{fg}$ | $8.56 \pm 0.42^{h}$ | $22 \pm 0.25^{ad}$ |
| B20 | $8.65 \pm 0.58^{a}$ | $6.19 \pm 0.66^{g}$ | $5.75 \pm 0.33^{ac}$ | $7.32 \pm 0.61^{i}$ | $28 \pm 0.46^{bef}$ |
| B21 | $8.43 \pm 0.75^{h}$ | $5.57 \pm 0.58^{h}$ | $6.72 \pm 0.54^{bd}$ | $5.43 \pm 0.25^{j}$ | $27 \pm 0.34^{b}$ |
| B27 | $8.32 \pm 0.65^{i}$ | $7.81 \pm 1.02^{i}$ | $5.87 \pm 0.35^{abcd}$ | $7.54 \pm 0.42^{d}$ | $23 \pm 0.84^{acf}$ |
| B34 | $8.79 \pm 0.84^{g}$ | $7.54 \pm 0.99^{e}$ | $6.6 \pm 0.47^{abd}$ | $6.68 \pm 0.48^{k}$ | $25 \pm 0.99^{cf}$ |
| B35 | $8.35 \pm 0.36^{i}$ | $6.25 \pm 0.47^{g}$ | $7.14 \pm 0.65^{d}$ | $5.74 \pm 0.61^{l}$ | $20 \pm 0.35^{d}$ |
| B38 | $8.74 \pm 0.24^{bdg}$ | $7.20 \pm 0.84^{f}$ | $5.47 \pm 0.66^{ef}$ | $7.18 \pm 0.63^{c}$ | $22 \pm 0.74^{ad}$ |

### 2.3 Probiotic properties of the selected lactobacilli strains

#### 2.3.1 Resistance to simulated gastrointestinal digestion

To provide their positive effects on the host's health, a probiotic must reach the intestine in large numbers and survive the stress factors present in the gastrointestinal tract (GIT). Therefore, the probiotic lactobacilli must have developed specific mechanisms to resist the harmful action caused by gastric acidity, bile salts, pepsin, pancreatin and other enzymes and antimicrobial compounds found in the GIT (Bendali *et al.*, 2011; Teles Santos *et al.*, 2016).

The results of tests measuring lactobacilli survival after exposure to simulated gastrointestinal conditions are summarized in Table 4. The 14 selected strains respondedto gastric and intestinal stress conditions differently and in a strain dependent manner.*Lactobacillus*B13 retained its viability during digestion with a decrease of CFU number of less than one logarithmic unit ($1.6x10^8$ CFU/mL comparing to the initial number of $4.3x10^8$CFU/mL). *Lactobacillus*strains B38 and B9 exhibited a decrease in viability level of one logarithmic unit while *Lactobacillus* B3 was more sensitive to this treatment (≈ 4.00 logs of loss).

The saliva-gastric step was the crucial phase which most significantly affected the lactobacillistrains survival ($p< 0.05$), whereas most of the strains were not affected by the bile chock and intestinal stepsand remained close to the initial population level. These findings arein agreement with those reported in previous studies (Bengoa *et al.*, 2017;Saito*et al.*, 2014; Teles Santos *et al.*, 2016). Indeed, it has been reported that some lactobacilli strains have high bile tolerance, stemming from their ability to produce the bile salt hydrolase (BSH) (Bendali *et al.*, 2011; Tulumoğlu*et al.*, 2014). Other studies have demonstrated that lactobacilli strains retained their viability when exposed to pH values of 2.5 - 4.0, but displayed loss of viability at pH values under 2.5 (Jacobsen *et al.*, 1999; Dunne *et al.*, 2001). In addition, the lower pH in fermented environments contributes to the acid tolerance of LAB strains (Guo*et al.,* 2015). The levels of resistance to saliva-gastric digestion, which have been observed in this work, were less pronounced than those reported by (Saito *et al.*, 2014); thisdifference could be due to the use of soy yogurt as a food matrix, which may confer cellsa certain amount of protection.

**Table 4.** Effect of the simulated gastrointestinal digestion on the viability of selected *Lactobacillus*strains

| Strains | Saliva-gastric digestion | | | Bile shock | | Intestinal digestion | |
|---|---|---|---|---|---|---|---|
| | 0 | 60 | 120 | 0 | 10 | 0 | 180 |
| B1 | 8.64 ± 0.55$^{ah}$ | 7.67 ± 0.25$^{a}$ | 6.50 ± 0.75$^{a}$ | 6.55 ± 0.25$^{a}$ | 6.51 ± 0.36$^{a}$ | 6.41 ± 0.33$^{a}$ | 6.22 ± 0.36$^{a}$ |
| B3 | 8.29 ± 0.47$^{b}$ | 6.76 ± 0.99$^{b}$ | 4.71 ± 0.44$^{b}$ | 4.76 ± 0.69$^{b}$ | 4.55 ± 0.52$^{b}$ | 4.50 ± 0.45$^{b}$ | 4.33 ± 0.74$^{b}$ |
| B5 | 8.53 ± 0.99$^{cd}$ | 6.81 ± 0.66$^{b}$ | 6.61 ± 0.63$^{c}$ | 6.63 ± 0.99$^{c}$ | 6.58 ± 0.99$^{a}$ | 6.49 ± 1.06$^{c}$ | 6.00 ± 0.98$^{ac}$ |
| B9 | 8.89 ± 0.22$^{e}$ | 7.74 ± 1.02$^{c}$ | 7.44 ± 0.85$^{d}$ | 7.50 ± 1.04$^{d}$ | 7.48 ± 0.57$^{c}$ | 7.34 ± 0.99$^{d}$ | 7.08 ± 0.99$^{d}$ |
| B13 | 8.63 ± 1.66$^{ah}$ | 8.56 ± 1.45$^{d}$ | 8.41 ± 0.25$^{e}$ | 8.46 ± 0.84$^{e}$ | 8.96 ± 0.35$^{d}$ | 8.81 ± 0.84$^{e}$ | 8.20 ± 0.84$^{e}$ |
| B16 | 8.75 ± 0.88$^{fj}$ | 8.11 ± 0.45$^{e}$ | 7.81 ± 0.75$^{f}$ | 7.36 ± 0.65$^{f}$ | 673 ± 0.48$^{e}$ | 6.08 ± 0.65$^{f}$ | 5.59 ± 0.25$^{f}$ |
| B18 | 8.60 ± 0.45$^{ch}$ | 7.40 ± 0.56$^{f}$ | 6.99 ± 0.66$^{g}$ | 6.65 ±0.47$^{cg}$ | 6.57 ± 0.24$^{a}$ | 6.71 ± 0.75$^{g}$ | 6.52 ± 0.36$^{c}$ |
| B19 | 8.52 ± 0.75d | 7.41 ± 0.32f | 6.77 ± 0.35$^{h}$ | 6.72 ± 0.35$^{g}$ | 6.62 ± 0.67$^{ae}$ | 6.49 ± 0.45$^{c}$ | 6.30 ± 0.14$^{ac}$ |
| B20 | 8.81 ± 0.66f$^{g}$ | 5.81 ± 0.65$^{g}$ | 5.86 ± 0.54$^{i}$ | 5.77 ± 0.48$^{h}$ | 5.73 ± 0.84$^{f}$ | 5.09 ± 0.33$^{h}$ | 5.12 ± 0.24$^{g}$ |
| B21 | 8.67 ± 0.56$^{ai}$ | 7.20 ± 0.47$^{h}$ | 5.47 ± 0.66$^{j}$ | 5.50 ± 0.17$^{i}$ | 5.53 ± 0.37$^{g}$ | 5.46 ± 0.54$^{i}$ | 5.27 ± 0.12$^{g}$ |
| B27 | 8.61 ± 0.47$^{ah}$ | 6.11 ± 1.11$^{i}$ | 5.34 ± 1.02$^{k}$ | 5.22 ± 0.36$^{j}$ | 5.19 ± 0.66$^{h}$ | 5.17 ± 0.35$^{j}$ | 5.01 ± 0.99$^{g}$ |
| B34 | 8.71 ± 0.44$^{ij}$ | 6.53 ± 0.43$^{j}$ | 5.38 ±0.35$^{jk}$ | 5.30 ± 0.48$^{k}$ | 5.35 ± 0.55$^{i}$ | 5.27 ± 0.14$^{k}$ | 5.15 ± 0.85$^{g}$ |
| B35 | 8.82 ± 0.66$^{g}$ | 7.61 ± 0.33$^{a}$ | 6.59 ±0.47$^{ac}$ | 6.45 ± 0.65$^{l}$ | 6.34 ± 0.47$^{j}$ | 6.33 ± 0.66$^{l}$ | 6.25 ± 0.45$^{ac}$ |
| B38 | 8.73 ± 0.24$^{ij}$ | 8.19 ± 0.45$^{k}$ | 7.33 ± 0.33$^{d}$ | 7.30 ± 0.74$^{f}$ | 7.28 ± 0.22$^{k}$ | 7.25 ±0.33$^{m}$ | 7.27 ± 0.36$^{d}$ |

### 2.3.2 Adhesion potential of the lactobacilli strains

The cell surface properties of hydrophobicity, auto-aggregation and co-aggregation appeared to be necessary for the adhesion of lactobacilli strains to epithelial cells and are essential for the colonization of the gastrointestinal tract.

**Auto-aggregation**

After 5 h of incubation, the tested lactobacilli strains showeda high auto-aggregating phenotype (Table 5). This phenotype exhibited a normal distribution, with the largest number of isolates being included in the interval between 40% and 70%. The lactobacilli strains B38 and B13 showed the highest auto-aggregation rate (72% and 68% respectively), while B21 showed the lowest (18%). These values were strongly strain dependent ($P < 0.05$). These parameter corroborate the results reported by (Caggia *et al.*, 2015). Such auto-aggregation may play an important role in preventing implantation ofpathogens and in eliminating them from the GIT. It is a consequence of complex physical and chemical interactions and larger and heavier cells will precipitate faster. However, the main factors that influence the ability to auto-aggregate may be the cell surface charge andcomposition(García-Cayuela *et al.*, 2014).

**Hydrophobicity**

In the present work, the cell surface hydrophobicity was measured using xylen as an apolar hydrocarbon (Table 5). The data showed that the mosttested lactobacilli displayeda good percentage of hydrophobicity ranged from 31% to 92% comparedto the minimum value (40%) reported by (Del Re*et al.,* 2000). The highest hydrophobic index was revealed by strainsB13, B38, B9, and B18 (≥80%), whereas B21, B3, and B27 exhibited the lowest percentages (≤ 40%). Our results are much better than those reported by (Abushelaibi*et al.*, 2017) who isolated lactic acid bacteria strainsfrom camel milk.

Cell surface hydrophobicity is a vital mechanism, mainly involved in the autoaggregation process. Previous studies revealed the existence of glycol-proteinaceous material that causes higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Kos *et al.*, 2003).

**Co-aggregation**

The co-aggregative phenotypes of lactobacilli were also investigatedwith two potential pathogens, namely *E. coli* and *S. aureus* (Table 5). Among the tested strains, B13 and B9 were the most co-aggregating strainswith respect to*E. coli*, whereas B38 and B13 were the most effective against*S. aureus*. These findings are better than those reported by(Solieri *et al.*, 2014). The co-aggregation of lactobacilli with pathogens is an interesting characteristic that which might form barriers and prevent host tissue colonization bypathogens (Del Re*et al.*, 2000).

**Table 5.** Hydrophobicity, autoaggregation and co-aggregation of the selected lactobacilli

| Strains | Hydrophobicity | Autoaggregation | Co-aggregation | |
|---|---|---|---|---|
| | | | *E. coli* | *S. aureus* |
| B1 | 4483 ± 1.02[a] | 43.15 ± 0.57[a] | 61.42 ± 1.55[a] | 52.65 ± 2.14[a] |
| B3 | 33.65 ± 0.56[b] | 21.03 ± 1.87[b] | 39.41 ± 1.32[b] | 34.55 ± 1.02[b] |
| B5 | 54.23 ± 1.44[c] | 41.08 ± 0.47[c] | 41.65 ± 0.47[c] | 47.65 ± 1.65[c] |
| B9 | 81.12 ± 1.58[d] | 61.42 ± 1.73[d] | 72.43 ± 1.22[d] | 60.24 ± 1.02[d] |
| B13 | 92.25 ± 1.98[e] | 68.63 ± 1.55[e] | 77.25 ± 2.45[e] | 63.44 ± 1.76[e] |
| B16 | 71.01 ± 2.01[f] | 52.43 ± 1.23[f] | 59.96 ± 1.66[a] | 61.23 ± 2.04[de] |
| B18 | 80.95 ± 1.57[d] | 48.65 ± 0.67[g] | 49.99 ± 1.27[f] | 37.76 ± 0.75[f] |
| B19 | 73.41 ± 1.42[fj] | 47.42 ± 0.66[h] | 47.56 ± 2.41[f] | 41.58 ± 1.58[g] |
| B20 | 57.09 ± 1.36[g] | 30.77 ± 1.44[i] | 44.21 ± 1.84[g] | 52.14 ± 1.64[ai] |
| B21 | 35.78 ± 1.58[h] | 18.76 ± 1.98[j] | 19.94 ± 1.94[h] | 25.21 ± 1.75[b] |
| B27 | 31.63 ± 1.74[b] | 22.80 ± 1.56[k] | 34.25 ± 1.55[i] | 30.24 ± 0.68[h] |
| B34 | 48.25 ± 2.63[i] | 53.56 ± 1.34[f] | 57.69 ± 1.36[j] | 42.52 ± 0.74[g] |
| B35 | 74.86 ± 1.03[j] | 45.89 ± 1.54[l] | 55.37 ± 1.85[k] | 50.44 ± 2.53[i] |
| B38 | 86.95 ± 2.34[k] | 72.86 ± 0.56[m] | 65.87 ± 1.66[l] | 67.24 ± 1.22[j] |

### 2.3.3 *In vitro* adhesion to HT-29 cell line

The capacity to adhere to epithelial cells and mucosal surfaces is another desirable attribute in probiotic strain selection, because it is closely related to their ability to colonize the gut and prevent their own immediate removal via peristalsis(Morelli, 2007). Adhesion to intestinal cells is reported to have beneficial health effects, including immunomodulation and the exclusion of pathogens by competing for binding sites and nutrients in the intestinal mucosa (Kravtsov *et al.*, 2008).

The adhesion rates of the 14 selected lactobacilli strains are presented in Fig. 1. The lactobacilli strains showed highly variable adhesion abilities, ranging from 27% to 84%. Strains B13, B9, and B38demonstrated the strongest adherence capacity 84%, 79%, and 74%, respectively. Our strains seemed to perform much better than previously described isolates(Colladoet *al.*, 2006).

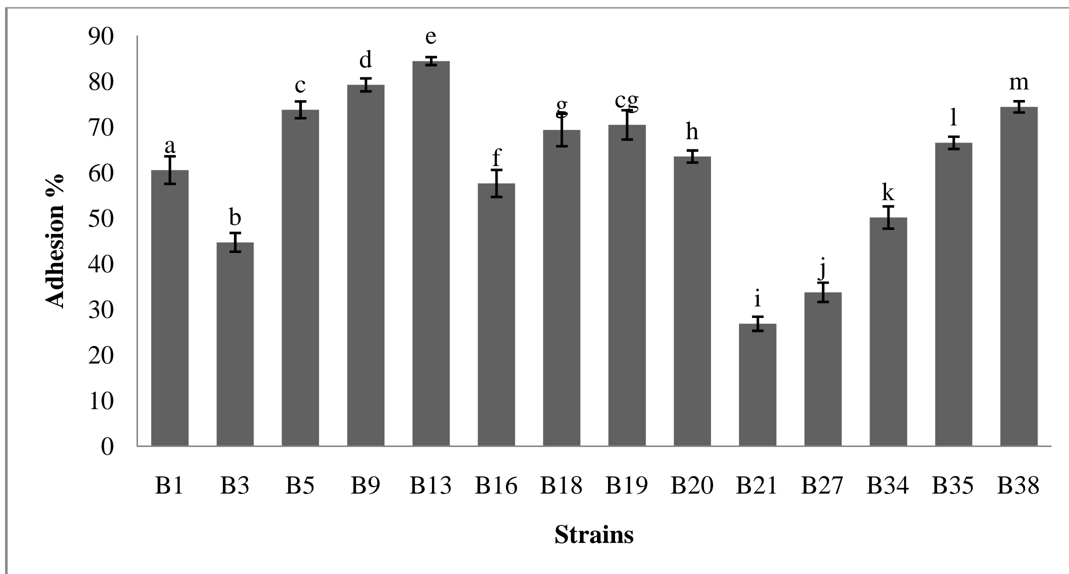


**Fig. 1.** Adhesion ability to the HT-29 cell line of the 14 selected lactobacilli strains

The adhesion of LAB to epithelial cells is both matrix and strain dependent, and varies within the same species (Duary, 2011). Cell adhesion is a complex process, which involves specific interactions between cell membranes and interacting surfaces (Han*et al.,* 2017). Kos *et al.* (2003) and García-Cayuela*et al.* (2014) found that the auto-aggregation and hydrophobicity properties of lactobacilli are necessary and crucial factors for their adhesion abilities. The results gathered in this work showed that those selected strains which displayed high percentages of auto-aggregation and hydrophobicity also manifested high adhesion capacities. This correlation is in accordance with previous data (Pithva *et al.*, 2014).

### 2.3.4 Antibiotics susceptibility

Probiotics are considered to be safe for human consumption, although their antibiotic resistanceis not necessarily a desirable trait because they can acquire and transfer genetic resistance to invading intestinal pathogens (Ocaña *et al.*, 2006). The lactobacilli strains were tested for their antibiotic sensitivity using the agar disc diffusion method. The results tabulated in Table 6 indicate that all isolated strainswere susceptible to rifampicin, tetracycline, chloramphenicol (except B5 and B20) and penicillin (except B5, B20, and B21). However, the strainsexhibited resistance to vancomycin, kanamycin and streptomycin (except B16 and B27).

Our findings corroborate previous results that were obtained using non-starter *Lactobacillus* strains isolated from ripened Parmigiano Reggiano cheese (Solieri*et al.*, 2014), and *Lactobacillus plantarum* Ln4 isolated from kimchi (Son *et al.*, 2017). In general, *Lactobacillus* strains exhibited resistance to amino-glycoside group antibiotics such as gentamycin, kanamycin, and streptomycin, a resistance which is not transferable to other species because it is chromosomally encoded (Danielsen & Wind, 2003).

**Table 6.** Antibiotics resistance of the lactobacilli strains

| Strains | P | V | K | C | T | S | R |
|---|---|---|---|---|---|---|---|
| B1 | S | R | R | S | S | R | S |
| B3 | S | R | R | S | S | R | S |
| B5 | R | R | R | R | S | R | S |
| B9 | S | R | R | S | S | R | S |
| B13 | S | R | R | S | S | R | S |
| B16 | S | R | R | S | S | MS | S |
| B18 | S | R | R | S | S | R | S |
| B19 | S | R | R | S | S | R | S |
| B20 | R | R | R | R | S | R | S |
| B21 | R | R | R | S | S | R | S |
| B27 | S | R | R | S | S | MS | S |
| B34 | S | R | R | S | S | R | S |
| B35 | S | R | R | S | S | R | S |
| B38 | S | R | R | S | S | R | S |

P: penicillin G, V: vancomycin, K: kanamycin, T: tetracycline, C: cloramphenicol, S: streptomycin, R: rifampicin.S: sensible, R: resistance, MS: moderately sensible.

Most of the strains showed sensitivity to penicillin, chloramphenicol, rifampin and tetracycline antibiotics, thus confirming the lower resistance of the lactobacilli species with respect to them(Vijayakumar *et al.*, 2015). Lactobacilli have been reported to possess natural resistance to vancomycin, which is attributed to the presence of D-Ala-D-lactate in their peptidoglycan, instead of the normal dipeptide D-Ala-D-Ala (Ashraf & Shah, 2011). On the other hand, antibiotic-resistant probiotic strains may benefit patients with unbalanced intestinal microbiota, or whose microbiota are greatly reduced in viable numbers due to the administration of a variety of antimicrobial agents (Salminen *et al.*, 1998).

### 2.3.5 Cholesterol removal

The ability to remove cholesterol has also been included amongst the selectioncriteria for probiotics. It has been proposed that lactobacilli exert hypocholesterolemic effects in different ways including assimilation, incorporation into cells' membranes, bile salt deconjugation (Li *et*

*al.*, 2012), and the production of short-chain fatty acids (De Preter *et al.*, 2007). The cholesterol removal abilities of the tested lactobacilli grown in MRS, supplemented with 0.3% bile salts, are illustrated in Fig. 2.

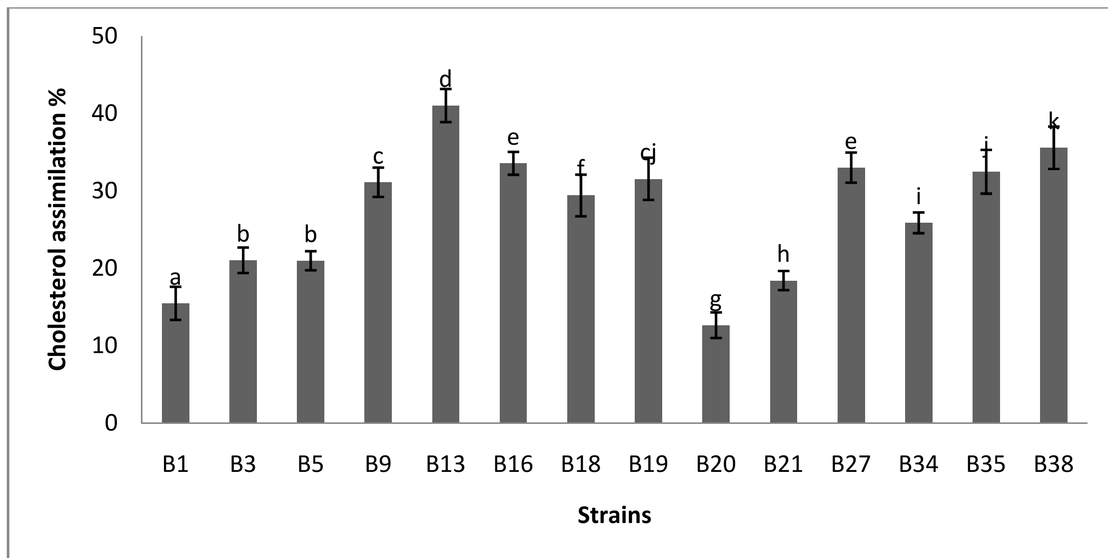


**Fig. 2.**Cholesterol percentage removal in the presence of Lactobacillus strains

All the strains were able to remove cholesterol to varying degrees. The amount of cholesterol assimilated by the cultures after 24 h of incubation ranged from 12.65% to 41.03%. Strain B13 manifested a superior ability (41.03%) to remove cholesterol from the medium, an ability which was significantly ($p < 0.05$) higher than that of the other examined lactobacilli strains. These levels of assimilation are close to those reported by (Shehata*et al.*, 2016) who isolated LAB strainsfrom different fermented milks. Further studies are required to determine the mechanism(s) involved in the removal of cholesterol by the tested strains.

### 2.3.6 Antioxidant activity of *Lactobacillus* strains

The lactic acid bacteria which colonize the intestine play a crucial role inprotecting the host from free radicals (Ren *et al.*, 2014). Oxidative damage is found to be associated with a variety of health disorders, such as cardiovascular diseases, diabetes, carcinogenesis, and ulcers

of the gastrointestinal tract (Li*et al.*, 2013). The free-radical-scavenging ability toward the DPPH was assessed in fig. 3.

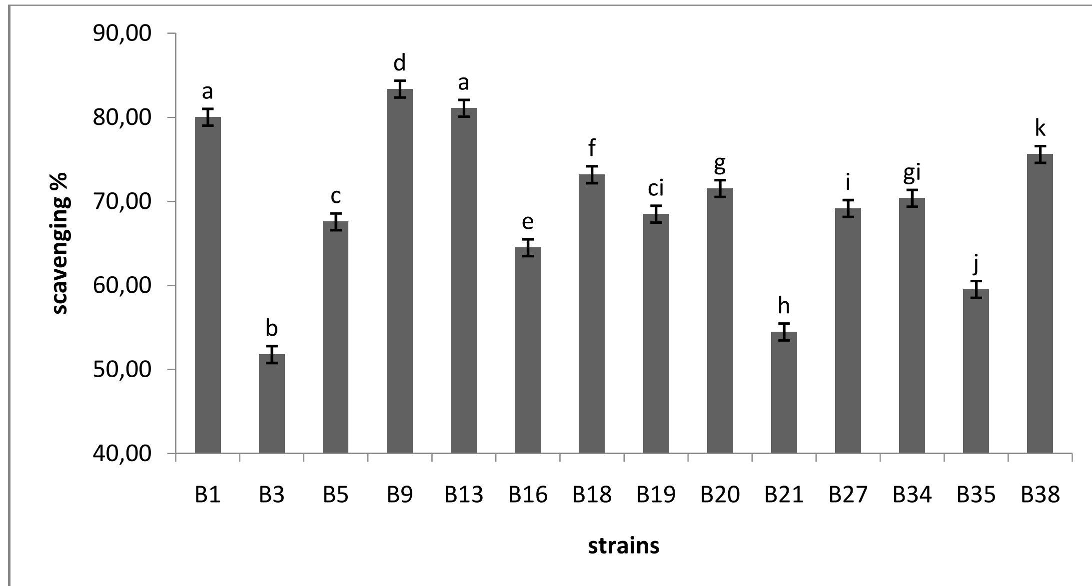


**Fig. 3.** DPPH Scavenging ability of the 14 selected lactobacilli.

The cell free supernatants of strainB9 exhibited maximum DPPH scavenging activity (83.37%) followed by strains B13 (81.09%), B1 (80.02%), and B38 (75.60%). These results are in agreement with earlier findings (Riaz Rajoka *et al.*, 2017). Several studies have demonstrated the involvement of lactobacilli strains in the decrease of oxidative stress and the accumulation of reactive oxygen species (ROS) (Forsyth, 2009; Xing *et al.*, 2015). (Bing, 1998) has reported that supernatant cultures of *Lactobacillus acidophilus* contains anti-oxidative metabolites. Similarly, *Lactobacillus plantarum* KCC-24, a strain isolated from an Italian rye-grass forage,has also displayed DPPH scavenging capacities (Vijayakumar *et al.*, 2015).

## 2.4 Identification of the selected strains

*Lactococcus* lactis C15 was identified by 16S rDNA as *Lactococcus lactis* subsp. *lactis* with an identity percentage ≥ 99% using BLAST. The Maldi Tof identification and 16S rDNA sequencing showed that B9, B13 and B38 belong to *Lactobacillus brevis* with a score of 2.3-2.5 and a homology of 99% respectively.

## 3 Conclusion

*Lactococcus lactis* subsp. *lactis* C15 is proposed as a potential starter culture for fermented milk mainly due to its high acidifying capacity and antibacterial activity. The 14 selected lactobacilli strains, which were isolated from artisanal Algerian cheeses revealed probiotic attributes. Thanks to their *in vitro* resistance to simulated gastrointestinal digestion, adhesion ability to intestinal cells, cholesterol removal and antioxidant activity, *Lactobacillus brevis* strain B13, followed by strains B38 and B9, showed the most promising results, making them interesting candidates for probiotic applications. However, further *in vitro* and *in vivo* studies are required to explore the health benefits of these strains before their use in new fermented foods developed with locally sourced strains and matrixes.

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## Chapter 2: Carob powder preparation and analysis

The results from this part were published as a manuscript in LWT-Food Science and Technology Journal.

# 1 Material and methods

## 1.1 Chemical and reagents

Human saliva α-amylase (14 µkat /mg proteins), pepsin from porcine gastric (11 µkat /mg), pancreatin from porcin pancreas (4xUPS, 0.12 µkat of trypsin/mg), α-amylase from *Bacillus licheniformis*, α-glucosidase and bile salts were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Solvents including acetone, methanol, diethyl ether, ethyl acetate, acetic acid, acetonitrile and HCl were analytical grade and purchased from Sigma-Aldrich. Folin-ciocalteau reagent, sodium carbonate, aluminium chloride,2, 2-dipheny-1-picryhydrazyl radical (DPPH), fluorescein, trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), 2, 2-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, DNS (3, 5-dinitrosalicylic acid), p-nitrophenyl-α-D-glucopyranoside and NaOH were obtained from Sigma-Aldrich (Oakville, ON, Canada). The phenolic acid standards (over ≥ 980 g/Kg pure); gallic, protocatechic, chlorogenic, caffeic, vanillic, syringic, p-coumaric, ferulic, o-coumaric, trans-cinnamic, the flavanoid standards; (+)-catechin, rutin, isoquercitrin, myricetin, apigenin and kaempferol were also purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

## 1.2 Sample preparation

Ripe carob (*Ceratonia siliqua* L.) pods were collected during July-August 2016 in Bejaia, Algeria. They were cleaned with distilled water and seeds were removed. The pulps were dried in microwave (Hotpoint Ariston, USA) at 720 W for 15 min (Talens*et al.*, 2016), ground to a fine powder using a commercial food blender and passed through a 0.149 mm sieve to obtain uniformly sized powder. The samples were stored at -20°C until analysis.

### 1.3 *In vitro* simulated gastrointestinal digestion

The static model proposed by (Minekus *et al.*, 2014) was followed to study the in-vitro digestion, including three sequential steps: oral, gastric and intestinal. Stock solutions; simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as the same molarity as reported in the method. All solutions were daily prepared and pre-warmed at 37°C before the use. Additionally, salivary α-amylase (mouth phase) was prepared in SSF to a final concentration of 1.25 µkat /mL, pepsin (stomach phase) in SGF to a final concentration of 33 µkat /mL as well as pancreatin (small intestine) in SIF to a final concentration of 2 µkat /mL (based on trypsin activity). In the mouth phase, eight gram (8 g) of carob pulp powder were transferred to 50 mL beaker and mixed thoroughly with 8.4 mL SSF solution, 1.2 mL of salivary α-amylase solution, 60 µL of 0.3 mol/L CaCl2 and 2340 µL of distilled water. Then, the obtained mixture was incubated in a water bath for 2 min at 37°C. Gastric digestion was continued by immediate addition of 7.5 mL of SGF, 5 µL of 0.3 mol/L $CaCl_2$, 695 µL of distilled water to the oral bolus and pH was adjusted to 3.0 with enough volume of 6 mol/L HCl. Next, 1.6 mL of porcine pepsin was added, and continuously kept under shaking (120 rpm) at 37°C for 2 h. Then, intestinal digestion was followed by the addition of 5.5 mL of SIF, 20 µL of 0.3 mol/L $CaCl_2$ and 1.25 mL of bile salts (25 mg/mL) to the mixture. After adjusting the pH to 7 with 6 mol/L NaOH, 2.5 mL of a pancreatin solution and 655 µL distilled water were added, kept under agitation (120 rpm) at 37°C for 2 h. Aliquots were collected at the end of each phase (oral, gastric and intestinal) and placed in an ice bath for 10 min to deactivate enzymes (Minekus, et al., 2014). Then, samples were freeze-dried (Labconco, Fisher Scientific, USA) and stored at - 20°C until further analysis.

### 1.4 Phenolic profiles

Soluble free, soluble conjugated and bound phenolics were extracted from digested and non-digested carob according to previously reported method (Krygier, et al., 1982), with some modifications. One gram of sample was extracted three times with 20 mL of 800 mL/L acetone at room temperature for 1 h under magnetic stirring. After centrifugation (Sorvall Legend XTR, Thermo Fisher Scientific, Germany) at 4000 g for 10 min, the combined supernatants were analyzed for soluble free and conjugated (esterified) phenolics while the residue was reserved for the determination of insoluble (bound) phenolics. The combined supernatants were evaporated

under vacuum using a rotary evaporator (Buchi R-215, Flawil, Switzerland) to remove the acetone and then acidified to pH 2.0.Free phenolics were extracted three times with diethyl ether-ethyl acetate (1:1, mL/mL). The organic phases were dehydrated with anhydrous sodium sulphate, combined and evaporated to dryness at 30°C. The aqueous phase obtained after free phenolic extraction was hydrolysed with 20 mL of 2 mol/L NaOH for 4 h at room temperature. The resultant hydrolysate was acidified to pH 2.0 and released conjugated (esterified) phenolics were extracted with diethyl ether-ethyl acetate as described above. For the bound phenolics extraction, the solid residue obtained from soluble free phenolics fraction was dissolved in 40 mL of 2 mol/L NaOH whilst stirring for 4 h. The mixture was then adjusted to pH 2.0, centrifuged and the insoluble-bound phenolics were extracted with diethyl ether-ethyl acetate (1:1, mL/mL) in the same manner as explained above. All samples were stored at -20°C until analysis.

## 1.5 Total phenolic content (TPC) and total flavonoid content (TFC)

The phenolics in both non-digested and digested samples were further analyzed for their TPC and TFC values as well as their antioxidant capacity. Firstly, phenolic compounds in each sample were extracted with 20 mL of 800 mL/L aqueous acetone containing 10 mL/L acetic acid for 3 h at room temperature. Thereafter, the mixture was centrifuged for 15 min at 4000 g and TPC was spectrophotometrically determined using the modified procedure of the folin-ciocalteu adapted to 96-well plate assay, as described by Gao *et al.* (2002). The absorbance was read at 725 nm using a microplate reader (Epoch, Biotek, Fisher Scientific, Winooski, USA) and expressed as mg of gallic acid equivalent per g of sample (mg GAE/g). TFC was also determined spectrophotometrically by following the procedure of Zhang et *al*. (2015), adapted to 96-well microplate (Costar, Corning Incorporated, Conrning, USA) assay. The absorbance was read at 515 nm and results were expressed as mg of Rutin equivalent per g of sample (mg RUE/g) (Zhang *et al*., 2015).

## 1.6 Antioxidant capacity

### DPPH

The antiradical activity of all samples was determined by DPPH (2,2-Diphenyl-1-picrylhydrazyl) (Brand-Williams*et al.*, 1995).Briefly, a solution of DPPH (1950 µl, 60 µM)

prepared in methanol was mixed with sample extract (50 µl). Distilled water was used as the control instead of extract. The reaction mixture was vortexed and left at 25°C in the dark for 30 min. Absorbance at 519 nm was measured with a spectrophotometer using methanol as a blank.Results were expressed as mg gallic acid equivalent per gram of sample (mg GAE/g) as follow:

DPPH % = 100 x (1- (absorbance of sample / absorbance of control)).

**ORAC**

Antioxidant activity was measured using the radical absorbance capacity (ORACFL) described previously by (Huang *et al.,* 2002).A multidetection microplate fluorescence reader (BioTek Instruments, Ottawa, ON, Canada) was used with excitation and emission wavelengths at 485 and 525 nm, respectively. Sample extracts and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) standards were diluted with 75 mM phosphate buffer (pH 7.4) prior to transfer into a 96-well microplate (Fluotrac 200, Greiner Bio-One Inc., Longwood, FL, USA). A peroxyl radical was generated by AAPH [2,2′azobis (2-methylpropionamide) dichloride] (Sigma-Aldrich, StLouis, MO, USA) during measurement, and fluoresceine was used as the substrate. Measurements were taken after 60 min at 37°Cupon addition of AAPH. Final ORAC values were calculated using a regression between the Trolox concentration (0-6 mg/ml) and the net area under the curve and expressed as mM Trolox equivalent (TE)/g sample.

**ABTS**

The ABTS•+ scavenging activity of all samples was conducted as described by Leite *et al.* (2011).ABTS•+ reagent was prepared by mixing 10 mL of 7 mM ABTS•+ (10 mL, 7 mM) with potassium persulfate (10 mL, 2.4 mM) and was kept in the dark at room temperature for 2 days. For analysis, 5 mL of ABTS•+ reagent was diluted in 100 mL of ethanol until the absorbance at 754 nm was approximately 0.7. Diluted ABTS•+ solution (2 mL) was added to each sample extract (50 µL) and mixed with ethanol (1.95 mL) to give a total volume of 4 mL. After standing at room temperature for 6 min, the absorbance at 754 nm was measured immediately using a spectrophotometer. Results were expressed as mg Trolox equivalents per g of sample (mg TE/g) (Leite *et al.*, 2011).

### 1.7 Phenolic content analyses (HPLC analyses)

Phenolic content analyses of non-digested and digested carob were performed by a reverse-phase (RP)-HPLC(Gunenc, HadiNezhad, Farah, Hashem, & Hosseinian, 2015). Samples were re-dissolved in methanol and filtered through 0.45 µm membrane PTFE filter, separated on an Alliance Waters 2695HPLC system (Waters Corp., Fisher Scientific, Milford, USA) equipped with a photodiode array detector (PDA, Waters 2998), Empower 3 software and auto sampler (Waters Corp., Milford, MA). Separations were carried out by means of an Atlantis R T3 column (150 mm x 4.6 mm, 5 µm particle size; Waters, Fisher Scientific, Milford, USA) using two solvents system: (A) 5 mL/L formic acid in milliQ water and (B) 100% acetonitrile. Phenolic compounds were analyzed at 30°C, injecting 10 µL of sample, using a gradient elution at 1 mL/min according to the following gradient program: 0 min, 95% A; 0- 35 min, 50% A; 35-40 min, 90% A and then return to 95% A in 10 min. The chromatograms were recorded at 280 for phenolic acids and 320 nm for flavonoids. The identification of the phenolic compounds was obtained by comparing the retention times with available external standards injected in the same conditions. Their quantification was carried out through calibration curves of the standards.

### 1.8 α-amylase inhibition assay

The α-amylase inhibition assay of both non-digested and digested carob was adapted from Telagari and Hullatti (2015) with some modifications. In brief, 50 µL of each sample at different concentrations (0.3, 0.5, 0.8 and 1 mg/mL) or negative control (distilled water) were pre-incubated with 10 µL of α-amylase (0.03 µkat /mL in 100 mmol/L sodium phosphate buffer pH 6.9) at 37°C for 20 min. Then, 20 µL of 1% soluble starch solution (dissolved in 100 mmol/L sodium phosphate buffer pH 6.9) was added to the mixture as a substrate and incubated for 30 min at 37°C. The reaction was stopped by the addition of 100 µL of DNS (3, 5-dinitrosalicylicacid) reagent and boiled for 10 min. Absorbance (Abs) was read at 540 nm in an Epoch microplate reader (Biotek,Epoch, Fisher Scientific, Winooski, USA). Percent inhibition was calculated relative to the negative control having 100% enzyme activity (Telagari & Hullatti, 2015) as follows:

$$\text{Inhibitory activity (\%)} = [(Abs_{sample} - Abs_{control})/Abs_{control}] * 100$$

### 1.9 α-glucosidase inhibition assay

The α-glucosidase inhibition assays of both non-digested and digested carob were measured according to the method of Yao *et al.*(2010). In a 96-well microplate, 50 µL of each sample at different concentrations (0.4, 0.5, 0.8 and 1 mg/mL) or negative control (distilled water) were reacted with 100 µL of α-glucosidase (0.03 µkat /mL dissolved in 100 mmol/L phosphate buffer, pH=6.9) and pre-incubated at 37°C for 20 min. Then, 50 µL of p-nitrophenyl-α-D-glucopyranoside (5 mmol/L in 100 mmol/L phosphate buffer, pH 6.9) was added to each well as a substrate and incubated at 37°C for 5 min. After incubation, absorbance (Abs) was recorded at 405 nm by a microplate reader (Yao *et al.* 2010). The α-glucosidase inhibitory activity was calculated as:

% inhibition = [(Abscontrol−Abssample)/Abscontrol] * 100.

### 1.10 *In vitro* fecal fermentation

The samples obtained after the intestinal digestion was centrifuged and the residue fraction (RF) was analyzed for colonic fermentation by growing the cultivable flora. Also, this part of the study was carried out in the Laboratoire de Microbiologie Appliquée (Bejaia, Algeria).

**Culture medium preparation**

The culture medium was prepared as described by Zhou *et al.* (2016). The composition for 1 L of nutrient medium was 2.0 g peptone, 2.0 g yeast extract, 0.5 g L-cysteine, 0.5 g bile salts, 0.1 g NaCl, 0.04 g $KH_2PO_4$, 0.01 g $MgSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2{\cdot}6H_2O$, 2 g $NaHCO_3$, 1.0 mL resazurin solution (1%, w/v), 2.0 mL Tween-80, and 10 µL vitamin K. The growth medium was sterilized by autoclaving at 121°C for 20 min (Zhou *et al.*, 2016).

**Fecal slurry and fermentation**

Fresh fecal samples were obtained from three healthy donors who reported no intestinal diseases and not received any antibiotic treatment during the last three months before the donation day. Samples were stored at 4°C and used within the 2 h of defecation. Fecal slurry was prepared by diluting feces in pre-sterilized phosphate buffered saline (PBS) solution (8 g/L NaCl,

0.2 g/L KCl, 1.15 g/L $Na_2HPO_4$ and 0.2 g/L $KH_2PO_4$, pH 7.3) to obtain 100 g/L fecal slurry to be used as the fermentation starter.

Then, 1 mL of the fecal slurry was added into 9 mL of culture medium containing 100 mg of RF obtained after the intestinal digestion step. Samples were incubated at 37°C in an anaerobic incubator. Aliquots were taken out after 15 min, 5 h and 24 h fermentation, and submerged in ice bath to halt microbial activity before HPLC analysis as described above.

### 1.11 Bioaccessibility index

The bioaccessibility index represents the amount of phenolic compounds released after simulated gastrointestinal digestion or fecal fermentation that could become available for absorption into the systemic circulation. This index was determined as follows:

$$\text{Bioaccessibility index (\%)} = (A / B) \times 100$$

Where: A is the total phenol content (µg) in samples after *in vitro* digestion or fecal fermentation and B is the total phenol content (µg) in samples before *in vitro* digestion or fecal fermentation.

### 1.12 Statistical analysis

Statistical analysis was performed using IBM's SPSS Statistic version 24 software. Results were shown as the mean ± standard deviation (SD) of triplicate experiments. The Kolmogorov-Smirnov test was applied to assess the normal distribution of the data. One-way analysis of variance (ANOVA) was applied for each parameter followed up with Tukey's post-hoc test for detecting significantly different means ($p < 0.05$).

## 2 Results and discussion

### 2.1 TPC and TFC of undigested carob

Total phenolic content (TPC) and total flavonoid content (TFC) of polyphenolic compounds (soluble free, soluble conjugated and bond) of undigested and *in vitro* digested carob samples were presented in Table 1.Both TPC and TFC values of undigested carob samples showed the predominance of bound phenolics. Indeed, the contribution of bound phenolics to TPC was 46% while soluble free and soluble conjugated phenolics contributed with 24% and 29%, respectively. Similar observations were also found in TFC with 36% contribution of bound

fraction while soluble free and soluble conjugated flavonoids contributed with 31% and 32%, respectively. Those results were expected since the literature reported that the most dominant phenolic compounds are abundantly present in plant in their bound form then their free form (de Camargo*et al.*, 2014). For instance, Wang *et al.*(2016) studied 14 selected bean samples and found the most phenolics in the bound fractions rather than free and conjugated forms. Similar results were also provided in the study of Gao *et al.* (2017) on seven commonly consumed vegetables in China. On the contrary, Sumczynski *et al.* (2016) showed a higher amount of TPC and TFC in the free fraction than the bound phenolics in commercial black and red rice.

## 2.2 TPC and TFC of *in vitro* digested carob

The *in vitro* gastrointestinal digestion is a widely used method to determine the bioavailability of ingested compounds. In the present study, the carob pulp was submitted to an in vitro simulation model of human digestion and fecal fermentation to determine the bioaccessibility and the bioactivity of its phenolic compounds in soluble free, soluble conjugated and insoluble bound forms. Changes in TPC and TFC contents of soluble free, soluble conjugated and bound carob phenolics at different digestion stages were also presented in Table 1. As can be observed, the *in vitro* digestion affected differently the content of phenolics and flavonoids in the three fractions.

A minimal change during oral phase ($p > 0.05$) was noticed comparing to the initial content (undigested carob), more specifically, only 12% of losses for TPC and 25% for TFC were recorded. These results were expected and could be explained by the low contact time of the mouth phase (2 minutes) and the marginal effects of α-amylase (1.25 µkat /mL) as mentioned by (Mosele*et al.*, 2016).

The gastric phase was deeply affecting ($p < 0.05$) both TPC and TFC of three fractional phenolics forms compared to undigested carob sample. The TPC values were decreased drastically by 45%, 38% and 56% in soluble free, soluble conjugated and bound fractions, correspondingly. Similarly, the TFC values were also decreased by 50% in free, 54% in conjugated and 49% in bound fractions.

**Table 1.** TPC and TFC of undigested and digested carob fractions: *soluble free, soluble conjugated and insoluble-bound**

| Analysis | Carob samples | Soluble Free | Soluble Conjugated | Bound | Total |
|---|---|---|---|---|---|
| TPC | Undigested | 15.4 ± 0.3[a] | 18.8 ± 0.1[a] | 29.1 ± 0.5[a] | 63.4 ± 0.3[a] |
| | Oral | 12.6 ± 0.4[a] | 16.0 ± 0.3[a] | 27.0 ± 0.4[a] | 55.7 ± 0.4[a] |
| | Gastric | 6.9 ± 0.4[b] | 7.2 ± 0.3[b] | 17.3 ± 0.2[b] | 31.5 ± 0.2[b] |
| | Intestinal | 11.1 ± 0.2[c] | 6.3 ± 0.4[b] | 9.3 ± 0.1[c] | 26.8 ± 0.2[c] |
| | **TPC (O+G+I)** | 30.6 ± 0.3 | 29.5 ± 0.3 | 53.6 ± 0.2 | 114.0 ± 0.3 |
| TFC | Undigested | 1.3 ± 0.7[a] | 1.4 ± 0.3[a] | 1.5 ± 0.3[a] | 4.3 ± 0.2[a] |
| | Oral | 0.8 ± 0.5[a] | 0.9 ± 0.1[a] | 1.4 ± 0.4[a] | 3.2 ± 0.3[a] |
| | Gastric | 0.6 ± 0.3[b] | 0.6 ± 0.1[b] | 0.8 ± 0.1[b] | 2.1 ± 0.1[b] |
| | Intestinal | 0.8 ± 0.5[a] | 0.5 ± 0.3[b] | 0.4 ± 0.2[c] | 1.8 ± 0.2[c] |
| | **TFC (O+G+I)** | 2.2 ± 0.3 | 2.0 ± 0.2 | 2.6 ± 0.2 | 7.1 ± 0.2 |

*Values are means of triplicates ± standard deviations and different letters in the same column indicate significant difference ($p < 0.05$) from Tukey's test. TPC (total phenolic content) values are milligram gallic acid equivalent per gram of sample (mg GAE/g sample): TFC (total flavonoid content) values are milligrams of rutin equivalent per gram of sample (mg RUE/g sample). O: oral phase, G: gastric phase, I: intestinal phase.

At the end of intestinal phase, a marked increase ($p < 0.05$) was observed in soluble free phenolic content (11.1 mg GAE/ g) and flavonoid content (0.8 RUE/g). This increase in both TPC and TFC suggests the main release of polyphenolic compounds from the carob powder in the intestinal digestion.The increases in the amount of free phenolic compounds after digestion were verified by many researches such in vegetables juices (Wootton-Beard *et al.*, 2011), cooked clove and nutmeg (Baker, Chohan, & Opara, 2013) and in persimmon fruit (Martínez-Las

Heras*et al.,* 2017). This phenomenon could be the result of intestinal digestive enzymes and bile salts acting on the food matrix, facilitating the release of bound phenolics to the digestive juice (Zhang *et al.*, 2017). On the other hand, these results are in contrast with the studies of Ortega *et al*. (2011) and Ydjedd *et al*. (2017) who reported important decrease in free phenolic compounds after gastrointestinal digestion of carob flour. This is probably due to major differences in the matrix, its preparation and the used protocols.

By contrast, soluble conjugated phenolic and flavonoids content showed a significant decrease ($p < 0.05$) under gastrointestinal conditions to reach values of 6.3 mg GAE/g and 0.5 RUE/g respectively. Same observation was recorded for the TPC and TFC in bound fraction which decreased respectively ($p < 0.05$) to 9.3 mg GAE/ g and 0.4 RUE/g compared with that before digestion (29.1 mg GAE/ g and 1.5 mg RUE/g).From these results, it can be concluded that the phenolic and flavonoid content insoluble conjugated and bound fractions tends to follow a different behavior than the free form along the digestion process of carob. The soluble conjugated and bound phenolic fraction demonstrated also different degree of decrease during digestion. These changes could be attributed to the release of conjugated and bound compounds from the carob under the effect of digestive enzymes.

### 2.3 Phenolics profile of undigested carob

The phenolics profile and their contents in soluble free, soluble conjugated and bound fractions of undigested carob were investigated by a RP-HPLC-DAD based on matching their retention time and UV absorbance spectra (280 and 320 nm) with respective standards. The results were represented in Table 2.Ten major phenolic acids corresponding to gallic, proto-catechuic, chlorogenic, vanillic, syringic, p-coumaric, caffeic, o-coumaric, ferulic and trans-cinnamic acids were detected in soluble (free and conjugated) and insoluble (bound) fractions of undigested carob. Similarly, six flavonoids were also found in these fractions and assigned to (+)-catechin, rutin, myricetin, isoquercitrin, apigenin and kaempferol.

**Table 2.** Phenolic profiles of undigested carob in soluble free, soluble conjugated and bound fractions*

| | Phenolic compounds | Soluble Free | Soluble Conjugated | Bound |
|---|---|---|---|---|
| Phenolic acids | Gallic acid | 162.5 ± 0.1[a] | 639.3 ± 0.1[b] | 1233.0 ± 0.1[c] |
| | Proto-catechuic acid | 39.2 ± 0.4[a] | 75.1 ± 0.1[b] | 226.0 ± 0.6[c] |
| | Chlorogenic acid | 54.1 ± 0.1[a] | 66.2 ± 0.6[a] | 523.3 ± 0.2[b] |
| | Vanillic acid | Nd | 7.4 ± 0.4[a] | 15.4 ± 0.4[a] |
| | Caffeic acid | Nd | 5.2 ± 0.1[a] | 14.3 ± 0.6[a] |
| | Syringic acid | Nd | 6.2 ± 0.9[a] | 16.0 ± 0.1[a] |
| | p-coumaric acid | 12.7 ± 0.1[a] | 24.7 ± 0.1[b] | 5.3 ± 0.2[c] |
| | Ferulic acid | 13.4 ± 0.1[a] | 117.5 ± 0.1[b] | 8.4 ± 0.1[a] |
| | o-coumaric acid | 11.0 ± 0.4[a] | 48.0 ± 0.1[b] | 142.4 ± 0.1[c] |
| | *Trans-* cinnamic acid | 42.4 ± 0.1[a] | 53.0 ± 0.6[a] | 82.0 ± 0.1[b] |
| | **Total phenolic acids (TPA)** | **335.3± 0.9[a]** | **1042.6 ± 0.4[b]** | **2266.1 ± 0.8[c]** |
| Flavonoids | (+)-catechin | 11.0 ± 0.3[a] | 24.4 ± 0.5[b] | 138.4 ± 0.2[c] |
| | Rutin | 118.3 ± 0.2[a] | 155.8 ± 0.1[b] | 89.1 ± 0.1[c] |
| | Myricetin | 18.1 ± 0.4[a] | 19.0 ± 0.1[a] | 8.2 ± 0.2[b] |
| | Isoquercitrin | 24.5 ± 0.6[a] | 42.8 ± 0.2[b] | 104.5 ± 0.4[c] |
| | Apigenin | 3.3 ± 0.8[a] | Nd | 10.6 ± 0.6[b] |
| | Kaempferol | Nd | Nd | 16.7 ± 0.1[a] |
| | **Total flavonoids (TF)** | **175.2 ± 0.1[a]** | **242.0 ± 0.1[b]** | **367.5 ± 0.1[c]** |
| | **Total phenolics (TPA+TF)** | **510.5 ± 0.2[a]** | **1284.6 ± 0.1[b]** | **2633.6 ± 0.2[c]** |

*Values are means of triplicates ± standard deviations (µg/g of sample) and different letters in the same row indicate significant difference ($p < 0.05$) from Tukey's test. Nd: not detected.

As observed earlier in TPC analysis (section 2.2.1), the bound form revealed higher ($p < 0.05$) amount of phenolics than the other two fractions with a total of 2633 µg/g of sample. Gallic acid was the most abundant phenolic acid in all carob fractions and its amount increased significantly ($p < 0.05$) after alkaline hydrolysis from 162.5 µg/g in free form to 1233 µg/g in bound form (Table 2). This finding was parallel to several authors (Benković*et al.*, 2017; Roseiro *et al.*, 2013) who reported the predominance of gallic acid in carob flour. No vanillic, caffeic or

syringic acid was detected in the free fraction, whereas they were released in the conjugated and bound fractions. With respect to flavonoids, rutin was mainly present in the free fraction whereas (+)-catechin and isoquercitrin were most detected in the soluble conjugated and bound fractions.

There is limited number of studies focused on the composition analysis of phenolics in carob plant (in free, conjugated and bound forms). To our knowledge, only Torun *et al*. (2013) conducted a study on the composition of free, ester and glycosides phenolics of natural and commercial carob flour. This same study reported the presence of six phenolic acids concentrated for most of them in the free fraction (Torun*et al.*, 2013).

## 2.4 Phenolics profile of digested carob and their bioaccessibility index

The effects of *in vitro* gastrointestinal digestion on bioaccessibility of carob phenolic compounds in the three fractions were evaluated in the present study and results are summarized in Table 3. Samples from each in vitro digestion steps were evaluated for their polyphenolic contents in soluble free, soluble conjugated and bound forms. In addition, depending on the available standards (mentioned in section 2.2.3), the main focus of this study was to measure only the free or hydrolysed forms of phenolics released after the in vitro digestion or the fecal fermentation.

Total phenolic acid (TPA) and total flavonoid (TF) contents of carob fractions after digestion showed similar trends as TPC and TFC results (Table 1). The polyphenolic content in the carob free fraction demonstrated a significant increase ($p < 0.05$) after the gastrointestinal digestion with reference to the initial carob values (Table 2). With regard to the individual phenolic, gallic and chlorogenic acids remained the most bioaccessible phenolic acids with percentages of 647.4% and 485.4% respectively. However, ferulic acid demonstrated a gradual

**Table 3.** Phenolic profiles of carob and their bioaccessibility during the *in vitro* gastrointestinal digestion*

| Fraction | Class | Phenolic compound | Oral | Gastric | Intestinal | Bioaccessibility (%) |
|---|---|---|---|---|---|---|
| Soluble free | Phenolic acids | Gallic acid | $152 \pm 8^{a}$ | $88 \pm 2^{b}$ | $984 \pm 16^{c}$ | 647.4 |
| | | Proto-catechuic acid | $32.1 \pm 0.4^{a}$ | $5.6 \pm 0.4^{b}$ | $113 \pm 14^{c}$ | 352.0 |
| | | Chlorogenic acid | $48 \pm 0.8^{a}$ | $15.2 \pm 0.3^{b}$ | $233 \pm 20^{c}$ | 485.4 |
| | | Vanillic acid | Nd | $4.2 \pm 0.9^{a}$ | $42 \pm 8^{b}$ | Nd |
| | | Caffeic acid | Nd | $1.7 \pm 0.5^{a}$ | $15 \pm 6^{b}$ | Nd |
| | | Syringic acid | Nd | $1.1 \pm 0.6^{a}$ | $53 \pm 7^{b}$ | Nd |
| | | p-coumaric acid | $11.9 \pm 0.6^{a}$ | $4.5 \pm 0.1^{b}$ | $46 \pm 15^{c}$ | 386.5 |
| | | Ferulic acid | $14.7 \pm 0.7^{a}$ | $10 \pm 1^{a}$ | $4.6 \pm 0.8^{b}$ | 31.2 |
| | | o-coumaric acid | $11.0 \pm 0.6^{a}$ | $10.2 \pm 0.7^{a}$ | $27 \pm 7^{b}$ | 245.4 |
| | | *Trans*- cinnamic acid | $39.4 \pm 0.6^{a}$ | $15.8 \pm 0.6^{b}$ | $49 \pm 11^{c}$ | 124.4 |
| | | **Total phenolic acids (TPA)** | $\mathbf{309 \pm 1^{a}}$ | $\mathbf{156 \pm 4^{b}}$ | $\mathbf{1566 \pm 19^{c}}$ | Nd |
| | Flavonoids | (+)-catechin | $12.0 \pm 0.4^{a}$ | $6.1 \pm 0.3^{b}$ | $67 \pm 21^{c}$ | 558.3 |
| | | Rutin | $110 \pm 10^{a}$ | $82 \pm 11^{b}$ | $294 \pm 17^{c}$ | 267.2 |
| | | Myricetin | $16 \pm 1^{a}$ | $5.5 \pm 0.1^{b}$ | $32 \pm 9^{c}$ | 200.0 |
| | | Isoquercitrin | $23.0 \pm 0.9^{a}$ | $16 \pm 2^{b}$ | $9 \pm 12^{c}$ | 39.1 |
| | | Apigenin | $3.3 \pm 0.4^{a}$ | $3.0 \pm 0.1^{a}$ | $8 \pm 6^{b}$ | 242.4 |
| | | Kaempferol | Nd | Nd | $12 \pm 7$ | Nd |
| | | **Total flavonoids (TF)** | $\mathbf{164 \pm 9^{a}}$ | $\mathbf{112 \pm 7^{b}}$ | $\mathbf{422 \pm 12^{c}}$ | **Nd** |
| | | **Total phenolics (TPA+TF)** | $\mathbf{473 \pm 10^{a}}$ | $\mathbf{268 \pm 9^{b}}$ | $\mathbf{1988 \pm 12^{c}}$ | **Nd** |
| Soluble Conjugated | Phenolic acids | Gallic acid | $616 \pm 24^{a}$ | $425 \pm 21^{b}$ | $331 \pm 18^{c}$ | 53.7 |
| | | Proto-catechuic acid | $64 \pm 11^{a}$ | $41 \pm 8^{b}$ | $18 \pm 1^{c}$ | 28.1 |
| | | Chlorogenic acid | $57 \pm 9^{a}$ | $27 \pm 5^{b}$ | $19 \pm 2^{c}$ | 33.3 |
| | | Vanillic acid | $6.0 \pm 0.1^{a}$ | $5 \pm 1^{a}$ | Nd | Nd |
| | | Caffeic acid | $4.4 \pm 0.1^{a}$ | $4 \pm 1^{a}$ | Nd | Nd |
| | | Syringic acid | $6.0 \pm 0.1^{a}$ | $5.0 \pm 0.1^{a}$ | $4 \pm 1^{a}$ | 66.6 |
| | | p-coumaric acid | $19.2 \pm 2.4^{a}$ | $9 \pm 0.1^{b}$ | $4.0 \pm 0.4^{c}$ | 21.0 |
| | | Ferulic acid | $109 \pm 7^{a}$ | $65 \pm 7^{b}$ | $28 \pm 4^{c}$ | 25.7 |
| | | o-coumaric acid | $43 \pm 9^{a}$ | $13 \pm 6^{b}$ | $4 \pm 0.1^{c}$ | 9.30 |
| | | *Trans*- cinnamic acid | $49 \pm 7^{a}$ | $34 \pm 10^{b}$ | $21 \pm 0.1^{c}$ | 42.8 |
| | | **Total phenolic acids (TPA)** | $\mathbf{974 \pm 10^{a}}$ | $\mathbf{628 \pm 7^{b}}$ | $\mathbf{429 \pm 8^{c}}$ | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Flavonoids | (+)-catechin | $18 \pm 3^{a}$ | $15 \pm 7^{a}$ | Nd | Nd |
| | | Rutin | $138 \pm 10^{a}$ | $102 \pm 18^{b}$ | $75 \pm 12^{c}$ | 54.3 |
| | | Myricetin | $17 \pm 11^{a}$ | $12 \pm 6^{b}$ | $4 \pm 0.5^{c}$ | 23.5 |
| | | Isoquercitrin | $40 \pm 4^{a}$ | $28 \pm 7^{b}$ | $17 \pm 1^{c}$ | 42.5 |
| | | Apigenin | Nd | $7.2 \pm 0.1$ | Nd | Nd |
| | | Kaempferol | Nd | Nd | Nd | Nd |
| | | **Total flavonoids (TF)** | **$213 \pm 11^{a}$** | **$164 \pm 10^{b}$** | **$96 \pm 9^{c}$** | |
| | | **Total phenolics (TPA+TF)** | **$1187 \pm 12^{a}$** | **$792 \pm 9^{b}$** | **$525 \pm 7^{c}$** | |
| **Insoluble bound** | Phenolic acids | Gallic acid | $1154 \pm 25^{a}$ | $854 \pm 22^{b}$ | $628 \pm 21^{c}$ | 54.5 |
| | | Proto-catechuic acid | $193 \pm 18^{a}$ | $98 \pm 15^{b}$ | $71 \pm 14^{c}$ | 36.8 |
| | | Chlorogenic acid | $487 \pm 20^{a}$ | $270 \pm 14^{b}$ | $185 \pm 10^{c}$ | 35.28 |
| | | Vanillic acid | $11 \pm 6^{a}$ | $6 \pm 1^{b}$ | Nd | Nd |
| | | Caffeic acid | $12 \pm 5^{a}$ | $5.0 \pm 0.1^{b}$ | Nd | Nd |
| | | Syringic acid | $15 \pm 5^{a}$ | $8.0 \pm 0.1^{b}$ | $7.0 \pm 0.1^{b}$ | 46.6 |
| | | p-coumaric acid | $3.2 \pm 0.6$ | Nd | Nd | Nd |
| | | Ferulic acid | $7 \pm 2^{a}$ | $4.0 \pm 0.6^{a}$ | $1.9 \pm 0.5^{b}$ | 27.1 |
| | | o-coumaric acid | $131 \pm 10^{a}$ | $108 \pm 11^{b}$ | $86 \pm 11^{c}$ | 65.6 |
| | | *Trans*- cinnamic acid | $70 \pm 7^{a}$ | $59 \pm 12^{b}$ | $33 \pm 8^{c}$ | 47.1 |
| | | **Total phenolic acids (TPA)** | **$2083 \pm 12^{a}$** | **$1412 \pm 11^{b}$** | **$1012 \pm 8^{c}$** | |
| | Flavonoids | (+)-catechin | $114 \pm 11^{a}$ | $71 \pm 8^{b}$ | $57 \pm 10^{c}$ | 50.0 |
| | | Rutin | $72 \pm 14^{a}$ | $61 \pm 10^{a}$ | $48 \pm 10^{b}$ | 66.7 |
| | | Myricetin | $2.4 \pm 0.1$ | Nd | Nd | Nd |
| | | Isoquercitrin | $88 \pm 12^{a}$ | $51 \pm 11^{b}$ | $30 \pm 1^{c}$ | 34.1 |
| | | Apigenin | $6.0 \pm 0.5$ | Nd | Nd | Nd |
| | | Kaempferol | $11 \pm 0.4$ | Nd | Nd | Nd |
| | | **Total flavonoids (TF)** | **$293 \pm 5^{a}$** | **$183 \pm 7^{b}$** | **$135 \pm 7^{c}$** | Nd |
| | | **Total phenolics (TPA+TF)** | **$2376 \pm 11^{a}$** | **$1595 \pm 12^{b}$** | **$1147 \pm 11^{c}$** | **Nd** |

*Values are means of triplicates ± standard deviations (µg/g of sample). Means followed by the different letters (a,b,c) in the same line represent a statistically significant difference ($p < 0.05$). Nd: not detected/determined.

reduce ($p < 0.05$) under the adopted conditions with only 31.2% of bioaccessibility. The increment in gallic acid concentration could be due to the hydrolysis of galloylated molecules as hydrolysable tannins (gallotannins) present in the carob matrix under *in vitro* digestion conditions. This result is supported by the study of Celep *et al.*(2017) who reported increment in the concentration of gallic and cholorogenic acid in *Hypericum perfoliatum* plant after intestinal digestion (Celep*et al.*, 2017). Also, another study (Farah*et al.,* 2008) reported the high bioaccessibilty of cholorogenic acid in green coffee and its rapid metabolism in humans.

The free flavonoids, (+)-catechin and rutin showed the highest bioaccessibility with 558.3% and 267.2%, respectively. This increase in the amount of flavonoids values is may be related to the hydrolysis of the complex compounds (as galloylated catechins) from their glycoside to aglycone forms (Ortega *et al.*, 2011). However, isoquercitrin was degraded gradually (39.1%). Same results were found by (Luzardo-Ocampo *et al.,* 2017) who reported a reduction in isoquercitrin concentration at the end of the intestinal phase in digestion of both corn and common bean chips. Also, another study (Ortega *et al.*, 2011) reported the complete degradation of isoquercitrin in carob flour. In another hand, the reason for some compounds having more than 100% bioaccessibility value is due to their increased amounts under the in-vitro digestion conditions compared to their free amount in the initial condition (before digestion). Indeed, the complex polyphenols both conjugated and bound (for example the gallotannins) of carob pulp will be released under the digestive enzymes activity. As a result, the amount of free phenolic will increase (i.e, gallic acid) and exceed the initial free amount quantified in the carob powder.

The conjugated and bound polyphenols were affected differently by the digestion conditions in each step. In fact, their total amounts (in mouth + gastric + intestinal phases) were decreased with a loss of 55.7% and 51.7%, respectively. Again, gallic acid remained the most abundant compound in both fractions while vanillic, caffeic, apigenin and kaempferol were not quantifiable after intestinal digestion. (+)-catechin and myricetin were also completely degraded in conjugated and bounds flavonoids respectively. These findings are in agreement with the work conducted by Juaniz *et al.* (2016) who reported a reduce in bound phenolics from 34% to 11% after the gastrointestinal digestion in raw green pepper (Juániz *et al.,* 2016). The same

authorsreported also the degradation of free and bounds polyphenols in raw and cooked cardoon when subjected to simulated digestion.

Also, it should be noted that, despite the decrease in concentration of bound phenolics during the gastrointestinal digestion, their degradation remains partial. In this regard, Adom & Liu (2002) reported that insoluble bound phenolics can resist the gastric and intestinal digestion to reach the large intestine since cell wall components are difficult to be digested (Adom & Liu, 2002). Also, Kroon *et al.*(1997) indicated that only 2.6% of ferulic acid was released from wheat under gastric and intestinal conditions.

## 2.5 Antioxidant capacity

In the present work, three different assays were carried out to measure the changes in carob antioxidant capacity induced during the digestion process. These methods were based on two different chemical mechanisms: the scavenging of the free radical DPPH and ABTS and oxygen radical absorbance capacity ORAC. Results illustrated in Table 4 represent the variation of antioxidant capacity in carob soluble free, esterified and insoluble-bound phenolic fractions before and after simulated gastrointestinal digestion stages.

DPPH, ABTS and ORAC values of carob phenolics in soluble free forms presented a great increase ($p < 0.05$) to reach respectively 107 mg GAE/g, 399 mg TE/g and 415 µmol TE/g under gastrointestinal conditions with reference to undigested extract. A similar trend (increase of the antioxidant capacity during gastrointestinal digestion) has been reported in the literature, for instance, DPPH and ABTS of different fruits were observed to be significantly increased after digestion process (Chen *et al.*, 2014). Likewise, a study of Pineda-Vadillo *et al.* (2016) published a high ORAC value of intestinal digested strawberry and peach yoghurt (Pineda-Vadillo *et al.*, 2016). However, other previous studies indicated a loss in antioxidant capacity after intestinal digestion (Gullon *et al.*, 2015; Burgos-Edwards *et al.*, 2017).

The higher antioxidant capacity observed after the digestive process could be attributed to pH changes and the deprotonation of the hydroxyl moieties present on the aromatic rings of the phenolic compounds (Bouayed*et al.*, 2011). It might be related also to the structural changes of phenolic molecules or liberation of new compounds having higher antioxidant capacity (Pineda-Vadillo *et al.*, 2016).

**Table 4.** Antioxidant capacity of soluble free, soluble conjugated and bound phenolics fractions of both undigested and digested carob*.

| Analysis | Carob samples | Soluble Free | Soluble Conjugated | Bound |
|---|---|---|---|---|
| DPPH | Undigested | 75 ± 7[a] | 102 ± 12[a] | 190 ± 10[a] |
| | Oral | 70 ± 10[a] | 107 ± 11[a] | 161 ± 8[b] |
| | Gastric | 60 ± 7[b] | 91 ± 8[a] | 84 ± 8[c] |
| | Intestinal | 107 ± 12[c] | 59 ± 8[b] | 66 ± 10[d] |
| ABTS | Undigested | 223 ± 10[a] | 365 ± 20[a] | 414 ± 12[a] |
| | Oral | 194 ± 9[a] | 315 ± 10[a] | 386 ± 12[a] |
| | Gastric | 134 ± 9[b] | 289 ± 14[a] | 277 ± 9[b] |
| | Intestinal | 399 ± 9[c] | 79 ± 11[b] | 89 ± 7[c] |
| ORAC | Undigested | 315 ± 12[a] | 419 ± 21[a] | 494 ± 14[a] |
| | Oral | 277 ± 11[a] | 354 ± 14[b] | 389 ± 12[b] |
| | Gastric | 168 ± 11[b] | 203 ± 8[c] | 246 ± 10[c] |
| | Intestinal | 415 ± 11[c] | 70 ± 7[d] | 102 ± 11[d] |

*Values are means of triplicates ± standard deviation (SD). Means followed by the different letters within a column are significantly different ($p < 0.05$). DPPH are expressed as mg Gallic equivalents/g; ORAC are expressed as µmol Trolox equivalents/g; ABTS are expressed as mg Trolox equivalents/g.

Antioxidant capacity in esterified and bound fractions changed differently during the digestion process. No significant differences ($p > 0.05$) was found in DPPH and ABTS values released from conjugated form after oral and gastric stages whereas a significant decrease ($p < 0.05$) was observed after intestinal phase (59 mg GAE/g and 79 mg TE/g). The ORAC values in conjugated form decreased gradually during the gastrointestinal simulation reaching 70 µmol TE/g. The same trend of decrease was also seen following the gastrointestinal digestion in the antioxidant capacity of bound fraction.

The results obtained in this work confirmed that carob extract containing high phenolic and flavonoid amounts had strongest antioxidant capacity. Indeed, several studies from scientific

literature reported a relationship between polyphenolic compounds and antioxidant capacity. Coefficients of correlation (r) were calculated to explain the relationship between TPC and TFC of carob fractions and their antioxidant values (DPPH, ABTS and ORAC).

In oral digestion, the three carob fractions showed positive and strong correlation ($r > 0.994$) between TPC and TFC and the antioxidant capacity measured with DPPH, ABTS and ORAC assays. The results obtained in gastric phase demonstrated a good correlation between TPC and the antioxidant capacity measured in free, conjugated and insoluble bound fractions. However, this correlation was moderate in case of TFC. At the end of intestinal digestion, again the DPPH, ABTS and ORAC values found in the three carob fractions were strongly consistent with TPC and TFC. These results are parallel to several previous studies (Gullon *et al.*, 2015; Lucas-Gonzalez *et al.*, 2016) which reported a high correlation between polyphenolic compounds and antioxidant capacity.

### 2.6 Fecal fermentation

Undigested polyphenols particularly those covalently bound to food matrix resist to digestive enzymes and reach the large intestine where they could be metabolized by colonic microbiota to become available for absorption. In the present work, the residual fraction (RF) of carob samples obtained after gastrointestinal digestion was submitted to fecal fermentation. The amount (µg/g of sample) and bioaccessibility percentage of polyphenols released during this process are listed in Table 5. Results showed an important microbial metabolic activity; the concentration of main phenolic compounds demonstrated a gradual increase during the first 5h of fermentation, whilst their amounts were rapidly degraded after 24h. Myricetin (79.5%) and gallic acid (20.0%) were the most abundant metabolites. The minor phenolics (e.g. vanillic and caffeic acids, kaempferol) were completely metabolized at the end of incubation period since they were notdetected.

**Table 5.** Phenolic compounds contents in carob (indigested fraction) during fecal fermentation*

| | Phenolic compounds | Control[1] | 15 min | 5 h | 24 h | Bioaccessibility (%) |
|---|---|---|---|---|---|---|
| Phenolic acids | Gallic acid | 641 ± 10[a] | 686 ± 11[b] | 711 ± 9[c] | 128 ± 4[d] | 20.0 |
| | Proto-catechuic acid | 143 ± 0.6[a] | 151.0 ± 0.1[a] | 86.0 ± 0.1[b] | 22.0 ± 0.2[c] | 15.4 |
| | Chlorogenic acid | 550 ± 12[a] | 570 ± 10[a] | 601 ± 10[b] | 68 ± 5[c] | 12.3 |
| | Vanillic acid | 31.0 ± 0.4 | Nd | Nd | Nd | Nd |
| | Caffeic acid | 10 ± 1[a] | 0.5 ± 0.1[b] | Nd | Nd | Nd |
| | Syringic acid | 20 ± 2[a] | 2.4 ± 0.1[b] | Nd | Nd | Nd |
| | p-coumaric acid | 34 ± 4[a] | 20.3 ± 0.1[b] | 8.7 ± 0.5[c] | Nd | Nd |
| | Ferulic acid | 14.4 ± 0.5[a] | 8.7 ± 0.8[b] | Nd | Nd | Nd |
| | o-coumaric acid | Nd | 6.7 ± 0.1[a] | 7.5 ± 0.1[a] | 10 ± 0.1[a] | Nd |
| | *Trans*- cinnamic acid | 11.7 ± 0.2 | Nd | Nd | Nd | Nd |
| | **Total phenolic acids (TPA)** | **1455 ± 12[a]** | **1446 ± 10[a]** | **1414 ± 9[b]** | **228 ±9[c]** | |
| Flavonoids | (+)-catechin | 60 ± 0.4[a] | 71 ± 0.1[a] | 30.1 ± 0.1[b] | Nd | Nd |
| | Rutin | 104 ± 0.2[a] | 62.0 ± 0.1[b] | 41 ± 0.1[c] | 20.0 ± 0.1[d] | 19.2 |
| | Myricetin | 20.5 ± 0.1[a] | 35 ± 0.1[a] | 41.5 ± 0.1[a] | 16.3 ± 0.1[b] | 79.5 |
| | Isoquercitrin | 42 ± 6[a] | 26 ± 1[b] | Nd | Nd | Nd |
| | Apigenin | Nd | Nd | Nd | Nd | Nd |
| | Kaempferol | 0.05 ± 0.14 | Nd | Nd | Nd | Nd |
| | **Total Flavonoid (TF)** | **226 ± 10[a]** | **194 ± 9[b]** | **112 ± 8[c]** | **36 ± 4[d]** | |
| | **Total phenolics (TPA+TF)** | **1681 ± 11[a]** | **1640 ± 8[b]** | **1526 ± 9.47[c]** | **264 ± 6[d]** | |

*,[1]: indigested fraction obtained after the gastrointestinal digestion of carob pulp. Different letters for each row indicate significant differences ($p < 0.05$). Nd: not detected/determined. Values are means of triplicates ± standard deviations (µg/g of sample).

This is consistent with the recent finding of Mosele *et al.* (2016) who reported an accumulation of gallic acid at early stages of incubation (2 h) and then its total degradation (24 h). Similarly, Alqurashi *et al.* (2017) reported also the decrease or the complete degradation of phenolic acids in acai after 24 h of fermentation. Juániz *et al.* (2016) found also a quick metabolization of isoquercitrin and its derivatives during the first minutes of the colonic biotransformation of raw green pepper.

The metabolism of polyphenols, in particular the bounds one in the colon is due to enzymatic release (esterase and xylanase activities) of the existing microflora (Acosta-Estrada*et al.,* 2014). In parallel, some new peaks (not identified because of unavailable standards) were observed during the fecal fermentation probably due to the generation of new microbial metabolites.

### 2.7 α- amylase and α-glucosidase inhibitory activities

To the best of our knowledge, no studies have compared the anti-diabetic activities in carob during the gastrointestinal digestion. Fig. 1 and 2 displayed the inhibitory effect of carob powder and its digested fractions on α- amylase and α-glucosidase activities.

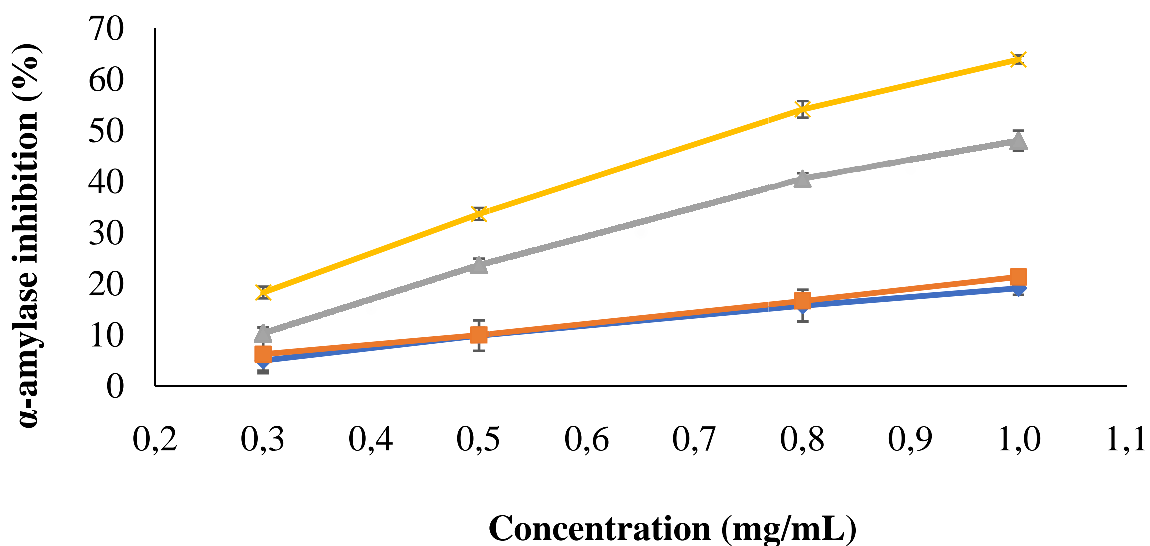


**Fig 1.** Effects of different concentrations (0.3, 0.5, 0.8, and 1 mg/mL) of undigested carob and its *in-vitro* digested fractions on α-amylase inhibitory activities. (Legend: ♦ Undigested carob, ■ oral fraction, ▲Gastric fraction, × Intestinal fraction).

It is clear that the gastric and intestinal fractions were more active and increased significantly the α-amylase inhibitory activity of carob. Both undigested carob and its digested fractions showed a positive dose-dependent inhibition of α-amylase activity (0.3-1.0 mg/mL). No significant differences ($p > 0.05$) in α-amylase inhibitory activity were observed between oral digestion and undigested carob. The intestinal fraction demonstrated the highest α- amylase inhibition with a rate of 64% at the concentration of 1 mg/mL. In this way, Silva *et al.* (2017) studied the inhibitory effect of polyphenols from guaraná and reported a rate > 60% after digestion. This might be due to the mechanism of inhibition by the capacity of polyphenols to bind and precipitate digestive enzymes as explained in a previous study (He*et al.*, 2007).

The α-glucosidase inhibitory of phenolic compounds was also increased significantly after gastric and intestinal digestion, but this activity was not linear to phenolics concentration. In other words, the percentage of inhibition decreases when the concentration of phenolic extract is high. Similar trend was observed in the study of Silva *et al.*(2017) who reported that the effect of guaraná extract on α-glucosidase activity was not positively related to its concentration.

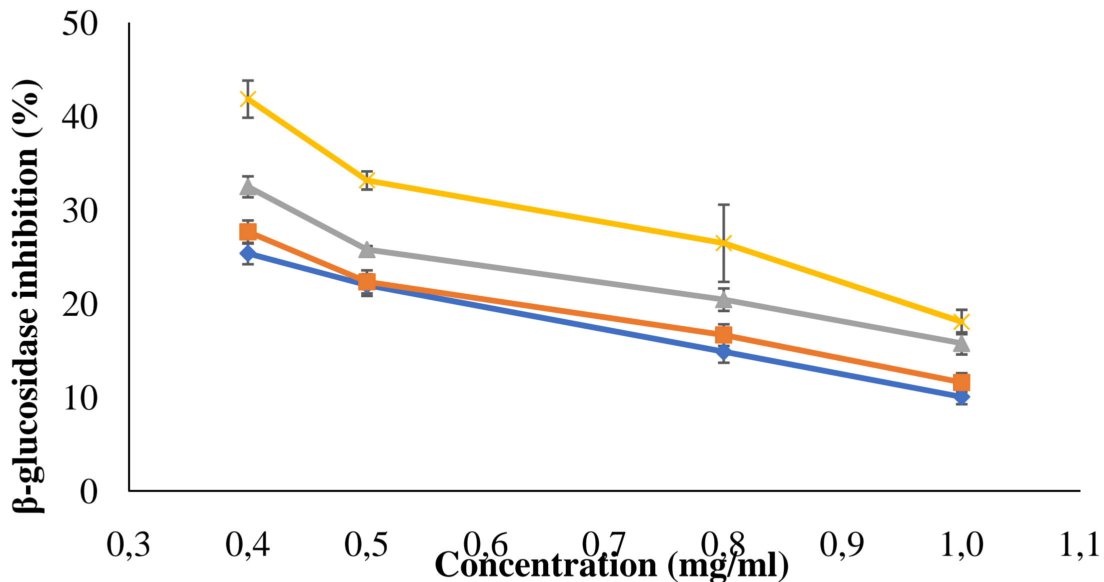


**Fig 2.** Effects of different concentrations (0.4, 0.5, 0.8, and 1 mg/mL) of undigested carob and its *in-vitro* digested fractions on α-glucosidase inhibitory activities. (Legend: Undigested carob, oral fraction, Gastric fraction, Intestinal fraction).

Again, the intestinal fraction exhibited stronger inhibitory effect with a percentage of 42% at the concentration of 0.4 mg/mL. These results were lower than those reported by Silva *et al.*(2017) who found 90% inhibition of guaraná extract for the same concentration.

As mentioned above, the in vitro digestion can affect greatly the content and ingredients of the food matrix. Thus, it can induce great contribution to inhibitory activity of α-amylase and α-glucosidase. The high inhibitory activities of intestinal fractions found in our study coincide with the high polyphenolics content in these fractions (Table 1). This let to suggest that phenolic compounds identified in the carob after digestion might influence glucose metabolism by inhibiting carbohydrate digestion.

## 3 Conclusion

In summary, the phenolic compounds in free, conjugated and bound fractions of carob pulp were significantly affected by the *in vitro* gastrointestinal digestion. Indeed, free phenolics were highly increased while the conjugated and bound phenolics were partially degraded over time under digestion conditions. Meanwhile, these changes resulted in liberation of compounds with high antioxidant capacity and increasing ability to inhibit enzymes involved in carbohydrate metabolism. Moreover, many of carob compounds remained non digestible reaching colon where they are metabolized to exert their health benefits. The results from this work clearly extend the knowledge in conjugated and bound phenolics composition and the effect of the digestion process on the health promoting properties of carob. However, some limitations were noted when conducting this study as the very limited information in literature about carob phenolic profiles in soluble and non-soluble fraction during digestion or non-digested carob, as well as the limit of the available standards. Further studies (typically the use of mass spectrometric (MS) detection) are required to fully understand the chemical structure modification and identification of bioactive compounds released after the digestion and the metabolites resulted under the microbial fermentation specifically that the digested fractions had demonstrated highest inhibitory activity on α-amylase and α-glucosidase.

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## Chapter 3: Fermented milk formulation and analysis

This part of the thesis aimed to the formulation of a synbiotic dairy beverage using the probiotic strain *Lactobacillus brevis* and carob powder as prebiotic's source.The results of this chapter were published in Journal of Food Bioactives.

# 1 Material and methods

## 1.1 Chemicals

Human saliva α-amylase (14 µkat /mg proteins), pepsin from porcine stomach (11 µkat /mg), pancreatin from porcine pancreas (4xUPS, 0.12 µkat of trypsin/mg), α-amylase from *Bacillus licheniformis*, α-glucosidase and bile salts were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Solvents including acetone, methanol, acetic acid and acetonitrile were analytical grade and purchased from Sigma-Aldrich. Folin-ciocalteau reagent, sodium carbonate, 2, 2-dipheny-1-picryhydrazyl radical (DPPH), fluorescein, trolox (6-hydroxy-2, 5, 7,8-tetramethylchroman-2-carboxylic acid), 2,2-azobis (2-methylpropionamidine) dihydrochloride (AAPH), potassium persulfate, DNS (3, 5-dinitrosalicylic acid), p-nitrophenyl-α-D-glucopyranoside and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The phenolic acid standards (over ≥ 980 g/Kg pure); gallic, protocatechic, chlorogenic, caffeic, vanillic, syringic, *p*-coumaric, ferulic, *o*-coumaric, *trans*-cinnamic, the flavanoid standards; (+)-catechin, rutin, isoquercitrin, myricetin, apigenin and kaempherol were also purchased from Sigma-Aldrich (St. Louis, Missouri, USA). De Man, Rogosa and Sharp (MRS), M17 (broths and agars) and lactose used for the growth of the bacterial strains were purchased from (Sigma Aldrich, Canada).

## 1.2 Carob powder preparation

Carob pods (*Ceratonia siliqua* L.) used in this study were collected during July-August 2016 in Bejaia city (Algeria) and were in total maturity stage (ripe). All carob pods were washed with distilled water and seeds were removed. The pulps were dried in microwave (Hotpoint Ariston, USA) at 720 W for 15 min, ground to a fine powder using a commercial food blender

and passed through a 0.149 mm sieve to obtain uniformly sized powder. The powder was then sterilized under UV irradiation for 30 min.

## 1.3 Fermented milk

### 1.3.1 Milk preparation and bacterial cultures

The skim milk (SM) was prepared by dissolving no-fat powder milk (great value, Ottawa, Canada) in warm distilled water (37°C), leading to reconstituted milk with about 13% (w/v) solid content. SM base was thermally sterilized at 90°C for 10 min in a water bath and stored at 4°C for 24 h (Tavares et*al.,* 2016). *Lactococcus* (*Lc.*) *lactis* C15 (starter culture), *Lactobacillus* (*Lb.*) *brevis* B13 and B38strains (probiotic cultures)used in this study were previously isolated from Algerian artisanal cheeses. These strains were selected based on their technological and probiotic properties, as well as their ability to grow in the presence of carob powder. All strains were phenotypically and genotypically identified by DNAr 16S sequencing (McGill University and Génome Québec Innovation Centre). For preparation of *inocula*, the three strains were activated by two successive culture transfers in de Man, Rogosa and Sharp (MRS) or M17 broth (Sigma Aldrich, Canada) at 30°C for 18 h, then in sterilized reconstituted skim milk (SM) (30°C for 18 h).

### 1.3.2 Fermentation process and storage

Prior fermentation, three batches of SM were prepared and warmed to 30°C. The first two batches were fortified separately with 4% (w/v) of carob powder or inulin (the best concentration of carob powder to be added to the fermented milks without resulting in syneresis was preliminary determined as 4% w/v. The third batch was used as control without any prebiotic supplementation. Then, *Lc. lactis* strain C15 was added at 1% (v/v) corresponding to $10^7$ CFU/ mL to the three batches. Each batch was then divided into four equal portions, which were inoculated with 1% (v/v) corresponding to $10^5$ CFU/mL of each *Lb. brevis* probiotic strain (B13 or B38) separately or in mixture as summarized in Table 1. All fermented milks were incubated at 30°C for 16 h and then stored at 4°C for 28 days. The products were analyzed after fermentation and on the 1$^{st}$, 7$^{th}$, 14$^{th}$, 21$^{st}$ and 28$^{th}$ day of storage for microbiological and physicochemical characteristics.

**Table1.** Experimental design for carob powder addition in fermented milks.

| Formulation | Prebiotic | Sample coding |
|---|---|---|
| SM + *Lc. lactis* C15 | 0 | F |
| SM + *Lc. lactis* C15 + *Lb. brevis* B13 | 0 | FP1 |
| SM + *Lc. lactis* C15 + *Lb. brevis* B38 | 0 | FP2 |
| SM + *Lc. lactis* C15 + *Lb. brevis* B13 + *Lb. brevis* B38 | 0 | FP12 |
| SM + *Lc. lactis* C15 | C | FC |
| SM + *Lc. lactis* C15 + *Lb. brevis* B13 | C | FCP1 |
| SM + *Lc. lactis* C15 + *Lb. brevis* B38 | C | FCP2 |
| SM + *Lc. lactis* C15 + *Lb. brevis* B13 + *Lb. brevis* B38 | C | FCP12 |
| SM + *Lc. lactis* C15 | I | FI |
| SM + *Lc. lactis* C15 + *Lb. brevis* B13 | I | FIP1 |
| SM + *Lc. lactis* C15 + *Lb. brevis* B38 | I | FIP2 |
| SM + *Lc. lactis* C15 + *Lb. brevis* B13 + *Lb. brevis* B38 | I | FIP12 |

**SM**: skim milk, **F:** fermented milk with no prebiotic addition, **C:** carob powder, **I**: inulin, **FC**: fermented milk with carob powder, **FI**: fermented milk with Inulin, **P1**: probiotic 1 (*Lb. brevis* B13), **P2**: probiotic 2 (*Lb. brevis* B38), **P12**: P1 + P2.

### 1.4 Microbiological analysis

Starter and probiotic strains viability was determined in all samples, just after fermentation and during storage using the pour plate technique. Hence, 1 mL aliquot samples were removed and subjected to appropriate serial dilutions in 0.1% (w/v) peptone water. *Lc. lactis* C15 enumeration was performed in M17 agar supplemented with 0.1% (w/v) lactose and *Lb. brevis* B13 and B38 counts were determined in MRS agar (pH = 5.4). The plates were then incubated at 30°C for 48 h under aerobic conditions.

### 1.5 Physicochemical characterization

The pH of various samples was measured directly with a pH meter (Orion 2-star pH benchtop, Thermo scientific, USA). The titrable acidity (TA) was determined and expressed as g of lactic acid per 100 g of fermented milk. The viscosity of each sample was measured using a Brookfield viscosimeter (model DV-E, MA, USA). According to the microbiological and physicochemical analyses of the carob supplemented fermented milks, the formulation showing the best microbial growth of probiotic strains and best physicochemical parameters was chosen to continue following studies. Total phenolic contents (TPC), HPLC and antioxidant capacity analyses were performed for the selected formulation before and during the storage process and the *in-vitro* gastrointestinal digestion.

### 1.6 Total phenolic contents (TPC)

TPC of fermented milks was spectrophotometrically determined using the modified procedure of the folin-ciocalteu adapted to 96-well plate assay, as described by Gao *et al.* (2002). The absorbance was read at 725 nm using a microplate reader (Epoch, Biotek, USA) and expressed as mg of gallic acid equivalent per g of sample (mg GAE/g).

### 1.7 HPLC analysis

Phenolic composition analysis was forms was performed for the selected fermented milk using a reverse-phase (RP)-HPLC (Waters Corp., Fisher Scientific, Milford, USA) according to Gunenc*et al.* (2015). Chromatographic separation was carried out by a RP Atlantis R T3 column (150 mm x 4.6 mm, 5 µm particle size; Waters, Milford, MA) using two solvents system: (A) 0.5% (v/v) formic acid in milliQ water and (B) 100% acetonitrile, under the following conditions: 0 min, 95% A; 0- 35 min, 50% A; 35-40 min, 90% A and then return to 95% A in 10 min. The chromatograms were recorded at 280 and 320 nm for phenolic acids and flavonoids, respectively. The identification of the phenolic compounds was obtained by comparing the retention times with available external standards injected in the same conditions. Their quantification was carried out through calibration curves of the standards.

## 1.8 Antioxidant capacity

### 1.8.1 DPPH scavenging activity

The antiradical ability of the selected fermented milk was measured by DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay according to the method of Brand-William*s et al.*(1995).The results were expressed as mg gallic acid equivalent per gram of sample (mg GAE/g) after reading the absorbance at 519 nm after 30 min of incubation (see details in chapter 2).

### 1.8.2 Oxygen radical absorbance capacity (ORAC)

The antioxidant capacity of the selected fermented milks was also performed following an oxygen radical absorbance capacity (ORAC) assay using a fluorescence plate reader according to the procedure ofHuang*et al.* (2002). ORAC values of all samples were calculated using the differences of areas under the fluorescence decay curves between the blank and a sample. The results were expressed as micromole Trolox equivalents per gram of sample (µmol TE/g) (see details in chapter 2).

## 1.9 *In vitro* simulated gastrointestinal digestion

The selected formulation of fermented milk (FCP12) was subjected to an *in vitro* simulated gastrointestinal digestion to determine the effect of digestion on its phenolic content and its antioxidant capacity as well as on the starter and probiotic strains viability. For this, 10 g of fermented milk was digested following the model reported by (Minekus *et al.*, 2014), which is based on three sequential steps: salivary, gastric and intestinal digestions. Simulated fluids (salivary, gastric and intestinal) and enzymes solutions were prepared at the same molarities as reported in the method. Aliquots were collected at the end of each phase (oral, gastric and intestinal) and placed in an ice bath for 10 min to deactivate the enzymes. Then, samples were freeze-dried and stored at - 20°C until further analysis. Regarding the enumeration of starter and probiotic strains, aliquots were plated on appropriate media as described in section 2.3.

## 1.10 Bioaccessibility index

The bioaccessibility index of phenolic compounds released from the fermented milk after digestion was calculated as follows:

$$\text{Bioaccessibility index (\%)} = (A / B) \times 100$$

Where: A is the content (µg) of individual phenolic compounds quantified by HPLC after the *in vitro* digestion of the fermented milk and B is the content (µg) of phenolic compounds quantified in fermented milk before the *in vitro* digestion.

### 1.11 α-amylase inhibition assay

The α-amylase inhibition assay of the selected fermented milk was adapted from (Telagari & Hullatti, 2015) with some modifications. Absorbance (Abs) was read at 540 nm in an Epoch microplate reader (Biotek, USA) and percentage of inhibition was calculated relatively to the negative control having 100% enzyme activity as follows:

$$\text{Inhibitory activity (\%)} = [(Abs_{sample} - Abs_{control})/Abs_{control}] * 100$$

### 1.12 α-glucosidase inhibition assay

The α-glucosidase inhibition assay was conducted according to the method of Yao*et al.*(2010). The α-glucosidase inhibitory activity was calculated as follows:

$$\%\ \text{inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] * 100.$$

### 1.13 Statistical analyses

All statistical analyses were carried using IBM's SPSS Statistic version 24 software. Data were expressed as the mean ± standard deviation (SD) of triplicate experiments. One way analysis of variance (ANOVA) was applied for each parameter followed up with Tukey's post-hoc test for detecting significantly different means ($p < 0.05$).

## 2 Results and discussion

### 2.1 Viable cell counts of starter and probiotic strains

As the viability of probiotic organisms is considered a key parameter for developing probiotic foods, the viable cell counts of starter and probiotic strains were evaluated after fermentation and their stability were monitored over 28-days storage as illustrated in Table 2. *Lc. lactis* C15 population remained relatively stable (values ranged between 9.41 and 9.77 log CFU/g) in all fermented milk formulations, after fermentation and throughout the storage. This strain was not affected ($p > 0.05$) by probiotic co-culture and the presence of prebiotics (inulin or carob powder) over storage period (data not shown). After 16 h of fermentation, the milks

enriched with carob powder (FC) showed significantly ($p < 0.05$) higher counts of probiotics lactobacilli (B13 and B38) (8 log CFU/g) than the control (F) and inulin enriched- fermented milk (FI) as presented in the Table 2.

**Table 2.** Probiotic *Lb. brevis* (P1 and P2) viable counts (log CFU/mL) in fermented milks during storage period (4°C)*

| Fermented milks | Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|---|
| FP1 | $6.43 \pm 0.11^{aA}$ | $6.42 \pm 0.09^{aA}$ | $6.35 \pm 0.08^{abA}$ | $6.28 \pm 0.09^{bA}$ | $6.17 \pm 0.04^{cA}$ | $6.04 \pm 0.25^{dA}$ |
| FP2 | $6.39 \pm 0.09^{aA}$ | $6.40 \pm 0.10^{aA}$ | $6.38 \pm 0.12^{aA}$ | $6.21 \pm 0.10^{bA}$ | $6.01 \pm 0.04^{cA}$ | $5.92 \pm 0.05^{cA}$ |
| FP12 | $6.28 \pm 0.12^{aA}$ | $6.26 \pm 0.08^{aA}$ | $6.11 \pm 0.25^{bA}$ | $6.05 \pm 0.04^{bA}$ | $5.82 \pm 0.13^{cA}$ | $5.74 \pm 0.12^{cA}$ |
| FCP1 | $8.68 \pm 0.12^{aB}$ | $8.69 \pm 0.10^{aB}$ | $8.80 \pm 0.13^{bB}$ | $8.79 \pm 0.04^{bB}$ | $8.69 \pm 0.09^{bB}$ | $8.28 \pm 0.09^{cB}$ |
| FCP2 | $8.61 \pm 0.18^{aB}$ | $8.60 \pm 0.09^{aB}$ | $8.79 \pm 0.08^{bB}$ | $8.81 \pm 0.11^{bB}$ | $8.70 \pm 0.09^{bB}$ | $8.26 \pm 0.14^{cB}$ |
| FCP12 | $8.66 \pm 0.11^{aB}$ | $8.65 \pm 0.05^{aB}$ | $8.98 \pm 0.08^{bB}$ | $8.93 \pm 0.12^{bB}$ | $8.89 \pm 0.25^{bB}$ | $8.58 \pm 0.21^{cB}$ |
| FIP1 | $7.67 \pm 0.05^{aC}$ | $7.65 \pm 0.05^{aC}$ | $7.57 \pm 0.09^{aC}$ | $7.41 \pm 0.05^{abC}$ | $7.30 \pm 0.25^{bC}$ | $7.27 \pm 0.07^{bC}$ |
| FIP2 | $7.69 \pm 0.18^{aC}$ | $7.70 \pm 0.11^{aC}$ | $7.64 \pm 0.12^{aC}$ | $7.47 \pm 0.12^{bC}$ | $7.\ 26 \pm 0.07^{cC}$ | $6.81 \pm 0.14^{dC}$ |
| FP12 | $7.61 \pm 0.08^{aC}$ | $7.60 \pm 0.11^{aC}$ | $7.53 \pm 0.21^{aC}$ | $7.46 \pm 0.13^{aC}$ | $6.82 \pm 0.15^{bC}$ | $6.43 \pm 0.05^{cC}$ |

*Values expressed in log CFU/mL as mean ± standard deviation (n = 3).$^{A,B,C}$ Different capital letters in a column indicates significant differences between fermented milk supplemented with different prebiotics ($P < 0.05$); $^{a,b,c,d}$ Different lowercase letters in a row for each fermented milk denote significant differences during storage period ($P < 0.05$). **F:** fermented milk without prebiotic addition including P1/P2, **FC**: fermented milk with carob powder including P1/P2, **FI**: fermented milk with inulin powder including P1/P2.

During the cold storage of the fermented milks, carob powder improved and increased the viability of the lactobacilli strains in the early stage of storage (D7), after that they remained relatively stable up to D21 (Table 2). In D28, the count of both strains decreased by about 0.5 log CFU/g in the individual cultures (FCP1 and FCP2) as well as in their co-cultures (FCP12). In addition, *Lb. brevis* B13 and B38 demonstrated better survival ($p < 0.05$) under refrigerated storage in the presence of each other (co-culture), probably due to synergistic effects of each probiotic. Even though the decrease in lactobacilli number at the end of storage, the minimum dose recommended by the scientific community to confer potential health benefits ($10^6$ CFU/g of food) (Vasiljevic & Shah, 2008) was maintained in carob fermented milk through its shelf-life. These findings point out an *in vitro* synbiotic effect of carob powder upon the probiotic strains, which was in accordance with several authors reports, who tested different prebiotic matrices in fermented milks such as lentil, onion juice and green banana flour (Agil *et al.*, 2013; ; Li *et al.*, 2016; Batista *et al.*, 2017). According to the obtained results, it seems that the constituents of carob powder favored the *Lb. brevis* B13 and B38 growth. It was already reported that carob pulp contains high level of dietary fibers (Ortega*et al.*, 2011) which was demonstrated to have a prebiotic effect on lactic acid bacteria (LAB) viability (Carlson *et al.*, 2018). The carob dietary fibers may protect the probiotic strains and serve as a carbon source for their survival during storage. Other studies have also reported the presence of substantial amounts of polyphenols (Owen *et al.*, 2003) in carob pulp that may have a positive effect on probiotics growth in milk. De Souza *et al.* (2018) reported that the addition of grape pomace extracts (rich on polyphenolic compounds) to fermented skim milk promoted the growth of *Lb. acidophilus*(de Souza de Azevedo *et al.*, 2018). Also, the supplementation of cow's milk with phenolic compounds extracted from olive vegetable water increased the growth of LAB by 2.5 log CFU/mL (Servili *et al.,* 2011). In addition, the carob polyphenols are important antioxidant factors (Ait Chait *et al.*, 2020), which might scavenge hydrogen peroxide and protect probiotic strains during the fermentation and storage.

### 2.2 Physicochemical characterization of fermented milks

Changes in pH, titrable acidity (TA) and viscosity of the fermented milks during storage period at 4°C arepresented in Table 3.

**Table 3.** The changes of physicochemical properties of different fermented milks during refrigerated storage*

| Fermented milks | pH | | | Titrable acidity (%) | | | Viscosity (cP) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 14 | Day 28 | Day 1 | Day 14 | Day 28 | Day 1 | Day 14 | Day 28 |
| **FP1** | $4.71 \pm 0.10^{aA}$ | $4.56 \pm 0.02^{abA}$ | $4.49 \pm 0.07^{bA}$ | $0.51 \pm 0.12^{aA}$ | $0.59 \pm 0.05^{abA}$ | $0.67 \pm 0.10^{bA}$ | $1102 \pm 0.08^{aA}$ | $1167 \pm 0.15^{aA}$ | $1218 \pm 0.16^{bA}$ |
| **FP2** | $4.70 \pm 0.04^{aA}$ | $4.58 \pm 0.10^{bA}$ | $4.50 \pm 0.04^{bA}$ | $0.50 \pm 0.08^{aA}$ | $0.57 \pm 0.10^{aA}$ | $0.66 \pm 0.13^{aA}$ | $1104 \pm 0.06^{aA}$ | $1170 \pm 0.16^{aA}$ | $1217 \pm 0.12^{bA}$ |
| **FP12** | $4.69 \pm 0.03^{aA}$ | $4.58 \pm 0.11^{aA}$ | $4.42 \pm 0.11^{bA}$ | $0.52 \pm 0.04^{aA}$ | $0.61 \pm 0.12^{aA}$ | $0.69 \pm 0.08^{aA}$ | $1107 \pm 0.12^{aA}$ | $1178 \pm 0.12^{aA}$ | $1224 \pm 0.08^{bA}$ |
| **FCP1** | $4.57 \pm 0.03^{aB}$ | $4.46 \pm 0.07^{aB}$ | $4.35 \pm 0.03^{bB}$ | $0.69 \pm 0.08^{aB}$ | $0.80 \pm 0.08^{bB}$ | $0.96 \pm 0.04^{cB}$ | $1336 \pm 0.14^{aB}$ | $1368 \pm 0.09^{aB}$ | $1408 \pm 0.14^{bB}$ |
| **FCP2** | $4.58 \pm 0.04^{aB}$ | $4.48 \pm 0.05^{aB}$ | $4.38 \pm 0.03^{bB}$ | $0.69 \pm 0.10^{aB}$ | $0.79 \pm 0.10^{bB}$ | $0.91 \pm 0.13^{cB}$ | $1324 \pm 0.13^{aB}$ | $1360 \pm 0.16^{aB}$ | $1398 \pm 0.13^{bB}$ |
| **FCP12** | $4.50 \pm 0.03^{aB}$ | $4.41 \pm 0.03^{aB}$ | $4.33 \pm 0.10^{bB}$ | $0.79 \pm 0.06^{aB}$ | $0.87 \pm 0.12^{bB}$ | $1.10 \pm 0.20^{cB}$ | $1343 \pm 0.12^{aB}$ | $1373 \pm 0.15^{aB}$ | $1423 \pm 0.14^{bB}$ |
| **FIP1** | $4.60 \pm 0.05^{aC}$ | $4.52 \pm 0.06^{aA}$ | $4.41 \pm 0.08^{bA}$ | $0.56 \pm 0.10^{aA}$ | $0.76 \pm 0.09^{bB}$ | $0.83 \pm 0.08^{bC}$ | $1203 \pm 0.10^{aC}$ | $1271 \pm 0.08^{aC}$ | $1301 \pm 0.12^{bC}$ |
| **FIP2** | $4.61 \pm 0.04^{aC}$ | $4.55 \pm 0.12^{aA}$ | $4.43 \pm 0.07^{bA}$ | $0.54 \pm 0.10^{aA}$ | $0.75 \pm 0.05^{bB}$ | $0.81 \pm 0.05^{bC}$ | $1200 \pm 0.16^{aC}$ | $1268 \pm 0.14^{aC}$ | $1298 \pm 0.08^{bC}$ |
| **FP12** | $4.60 \pm 0.04^{aC}$ | $4.50 \pm 0.10^{aA}$ | $4.39 \pm 0.02^{bB}$ | $0.60 \pm 0.12^{aA}$ | $0.78 \pm 0.10^{bB}$ | $0.89 \pm 0.14^{bC}$ | $1218 \pm 0.14^{aC}$ | $1276 \pm 0.22^{bC}$ | $1324 \pm 0.07^{cC}$ |

*Values were expressed as mean ± standard deviation (n = 3). $^{A,B,C}$ Different capital letters in a column denote significant differences between fermented milk supplemented with different prebiotics ($P < 0.05$); $^{a,b,c}$ Different lowercase letters in a row for each fermented milk denote significant differences during storage period ($P < 0.05$). Abbreviations are: **F**: fermented milk without prebiotic addition including P1&P2, **FC**: fermented milk with carob powder including P1&P2, **FI**: fermented milk with inulin powder including P1&P2.

The addition of 4% (w/v) of carob powder promoted ($p < 0.05$) acids productionin the milks during fermentation comparatively to both control and milk containing inulin. Thus, the pH values of the skim milk (SM) (pH = 6.67) were declined significantly ($p < 0.05$) to about 4.5 while the TA values were increased to about 0.6%. Also, milks fermented with the combination of *Lb. brevis* B13 and B38 were more acidic than those containing the single strain ($p < 0.05$). The increase in acidity could be attributed to the metabolic activity of starter and probiotic cultures, which had the ability to produce organic acids from the compounds supplied by the carob powder as polysaccharides and polyphenols. The pH continued to drop and TA increased throughout the whole period of cold storage to stabilize during the last week to reach 4.3 and 0.9-1.0% respectively in carob fermented milk. Several previous studies have shown that the incorporation of prebiotics may reduce the pH values in fermented food with advanced storage (Boudjou*et al.*, 2014; Freire *et al*., 2017; Su*et al.*, 2018). Carob powder supplementation resulted in an increase of the viscosity of the fermented milks during the storage time to reach values between 1398 and 1423 cP at the end of the process. Again, the co-culture of the two probiotic strains led to more viscous milk than those fermented with the individual cultures. Our findings were in accordance with previous studies, who reported that addition of dietary fibers in fermented milk increase the viscosity of the final product (Güler-Akın*et al.*, 2016; Tavares Estevam *et al.*, 2018). This increase in viscosity was probably related to pH decrease which promotes the coagulation of milk and gel formation (Williams & Phillips, 2009). Thanks to the results obtained after the microbiological and physicochemical analyses, milk fermented with the combination of *Lb. brevis* B13-B38 and fortified with 4% (w/v) of carob powder (FCP12) was further characterized as indicated below.

### 2.3 Carob fermented milk during cold storage: TPC, polyphenolic profile and antioxidant capacity

**TPC:** Figure 1 (A) shows TPC values of the selected formulation of carob fermented milk (FCP12) determined after fermentation and at different points over the 28-days storage period. Compared to unsupplemented fermented milks (control), the incorporation of carob powder enhanced significantly the TPC in fermented milk following the fermentation to 5.48 mg gallic acid equivalent/g.

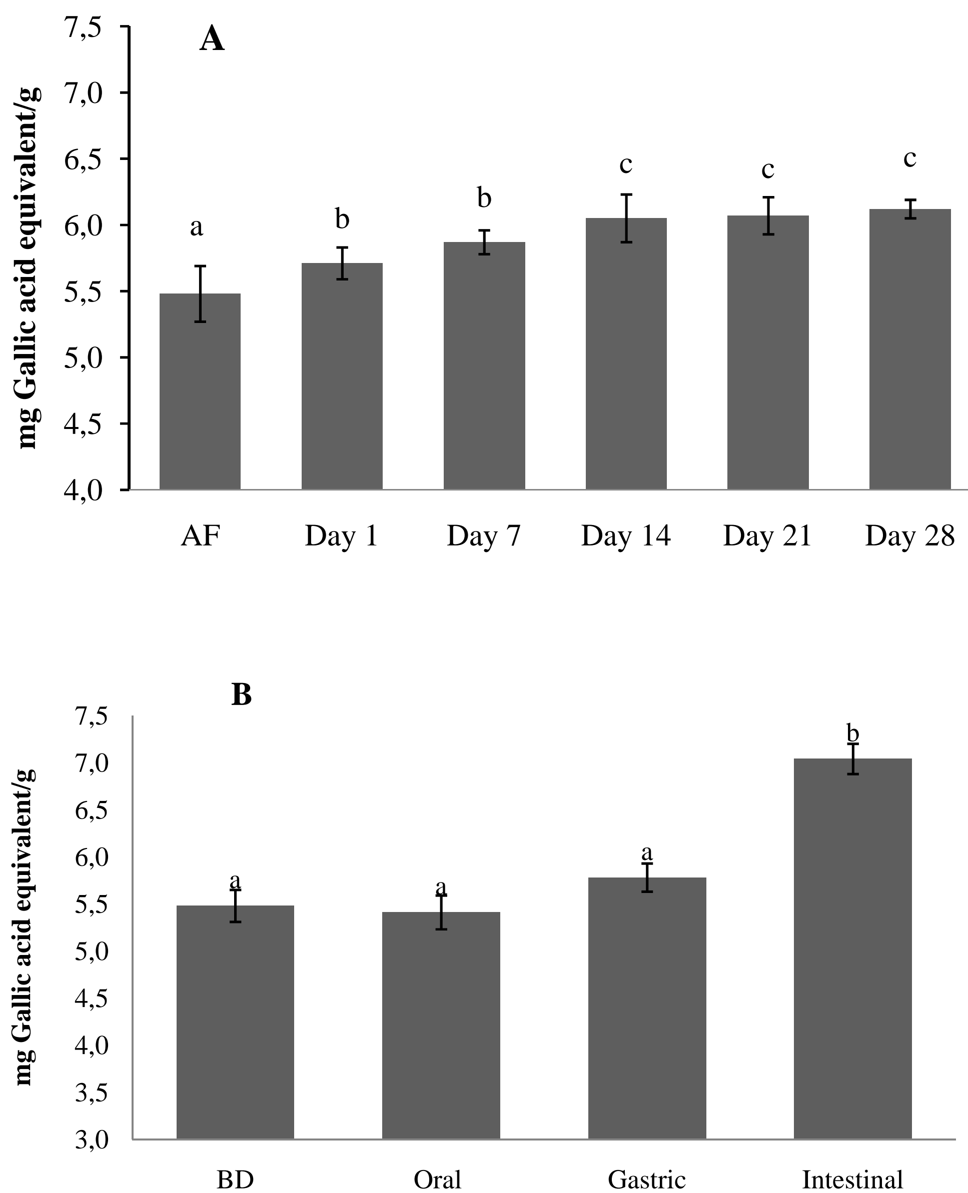


**Fig 1.**Changes in total phenolic content (TPC) of carob fermented milk (FCP12) during refrigerated storage (A) and *in vitro* digestion (B). Small letters indicate significant differences ($p < 0.05$) during storage period and different steps of digestion of the fermented milk. AF: After fermentation (16 h), BD: Before digestion.

The amount of phenolic compounds in control (1.12 mg gallic acid equivalent/g) was probably due to the presence of other compounds in milk other than polyphenols such as low molecular weight antioxidants, free amino acids, peptides and proteins (Helal & Tagliazucchi, 2018). The TPC in the fermented milk fortified with carob increased up to D14 and then remained stable in the course of the storage time. This increase could be due to the ability of LAB strains to hydrolyze the phytochemicals complex there by releasing soluble conjugated or insoluble bounded phenolic compounds from plant cell wall (Gan*et al.*, 2017; Kwaw *et al.*, 2018). In addition, proteolysis of milk proteins occurring during the fermentation process may release amino acids with phenolic side chains such as tyrosine and tryptophan (Korhonen, 2009). Similarly, Santos *et al.* (2017) reported an increase of TPC amount in fermented goat milk supplemented with grape pomace extract.

**Polyphenolic profile:** The polyphenolics compounds in carob powder and carob fermented milks (FCP12) were identified and quantified by HPLC and the results (Table 4) confirmed gallic and chlorogenic acid as the most representative phenolic acids in carob powder, whereas rutin and isoquercitrin were the most abundant flavonoids. Fermentation of the carob enriched-milk by starter and probiotic bacteria revealed interesting traits with regard to metabolization of polyphenolic compounds. As detected in carob powder, gallic acid was found at the highest concentration (102.35 µg/g) followed by chlorogenic acid (33.67 µg/g) in carob fermented milk (FCP12). Rutin and isoquercitrin were also the most quantified flavonoids. As expected, no phenolic acids and flavonoids were found in the unsupplemented fermented milk. On the contrary, proto-catechuic and ferulic acids as well as catechin behaved differently than the other phenolics and marked a significant ($p < 0.05$) decrease in their concentration. The concentrations of different phenolic compounds remained constant or even slightly increased ($p > 0.05$) over time refrigerated storage until D14 where the concentrations decreased significantly ($p < 0.05$) to total phenolic compounds of 205.66 µg/g in D28. As discussed above, the changes of individual phenolics found in carob fermented milk during fermentation and cold storage are due to their metabolization by the starter and probiotic strains. The study of Curiel *et al.* (2010) had investigated the potential of *Lb. brevis* strains to degrade food phenolic acids and reported that those strains can metabolize and decarboxylate some cinnamic acids (p-coumaric, ferulic and caffeic acids) to their corresponding vinyl derivatives (vinyl phenol, vinyl catechol, and vinyl guaiacol) via a phenolic acid decarboxylase enzyme (Curiel*et al.*, 2010).

**Table 4.** Phenolic compounds of carob fermented milk (FCP12) during refrigerated storage (4°C)*.

| Phenolic compounds | Carob powder | Day 0 | Day 1 | Day 14 | Day 28 |
|---|---|---|---|---|---|
| Gallic acid | 162.50 ± 0.12 | 102.35 ± 0.14[a] | 100.82 ± 0.17[a] | 112.41 ± 0.07[a] | 78.06 ± 0.10[b] |
| Proto-catechuic acid | 39.19 ± 0.04 | 24.71 ± 0.04[a] | 21.20 ± 0.08[a] | 19.64 ± 0.11[a] | 10.27 ± 0.09[a] |
| Chlorogenic acid | 54.12 ± 0.11 | 33.67 ± 0.24[a] | 34.18 ± 0.20[a] | 35.21 ± 0.12[a] | 26.14 ± 0.07[a] |
| Caffeic acid | Nd | Nd | Nd | Nd | Nd |
| p-coumaric acid | 12.77 ± 0.11 | 8.11 ± 0.18[a] | 8.10 ± 0.12[a] | 11.04 ± 0.06[a] | Nd |
| Ferulic acid | 13.44 ± 0.12 | 7.47 ± 0.14[a] | 7.89 ± 0.08[a] | 10.41 ± 0.07[a] | 3.75 ± 0.14[b] |
| o-coumaric acid | 11.07 ± 0.04 | 6.33 ± 0.20[a] | 5.47 ± 0.11[a] | 4.15 ± 0.08[a] | Nd |
| *Trans*- cinnamic acid | 42.40 ± 0.15 | 24.10 ± 0.11[a] | 24.85 ± 0.22[a] | 18.47 ± 0.07[a] | 10.63 ± 0.15[b] |
| **Total phenolic acids (TPA)** | **335.49 ± 0.18** | **206.74 ± 0.14[a]** | **202.51 ± 0.17[a]** | **211.33 ± 0.17[a]** | **128.85 ± 0.16[b]** |
| (+)-Catechin | 11.02 ± 0.03 | 8.23 ± 0.10[a] | 8.21 ± 0.11[a] | 9.04 ± 0.11[a] | Nd |
| Rutin | 118.36 ± 0.02 | 88.76 ± 0.15[a] | 89.06 ± 0.08[a] | 89.86 ± 0.08[a] | 64.07 ± 0.20[b] |
| Myricetin | 18.17 ± 0.04 | 10.53 ± 0.11[a] | 10.50 ± 0.14[a] | 8.12 ± 0.15[ab] | 5.17 ± 0.08[b] |
| Isoquercitrin | 24.47 ± 0.06 | 15.20 ± 0.14[a] | 15.18 ± 0.15[a] | 15.89 ± 0.14[a] | 7.57 ± 0.11[b] |
| **Total flavonoids (TF)** | **172.02 ± 0.14** | **122.72 ± 0.16[a]** | **122.95 ± 0.18[a]** | **122.91 ± 0.17[a]** | **76.81 ± 0.11[b]** |
| **Total phenolics (TPA+TF)** | **507.51 ± 0.23** | **329.46 ± 0.20[a]** | **325.46 ± 0.20[a]** | **334.24 ± 0.20[a]** | **205.66 ± 0.19[b]** |

*Values are means of triplicates ± standard deviations (µg/g of fermented milk sample). Means followed by the different letters (a,b) in the same row represent a statistically significant difference ($p < 0.05$). **Nd:** not detected.

According to the same authors, *Lb. brevis* can also convert gallic and protocatechuic acids to pyrogallol and catechol, respectively. Another explanation for the decrease of phenolic acids concentrations is the presence of milk proteins that can bind and precipitate carob polyphenols. Indeed, the acidic pH caused by the fermentation, may enhance the binding affinity between phenolic compounds and milk proteins (Helal & Tagliazucchi, 2018). Same authors found that the addition of 25% milk to a cinnamon beverage resulted in formation of insoluble complexes between cinnamon tannins and milk proteins, which decreased the total polyphenols content by 28%.

**Antioxidant capacity:** Since the antioxidant activity of food manifests itself with different mechanisms, a single chemical method may not determine the total antioxidant capacity. For this, the antioxidant capacity of carob fermented milk (FCP12) was evaluated in the present work by two different methods. The variations in DPPH and ORAC values of carob fermented milk are shown in Fig 2 (A, B). The inclusion of carob powder into milk had a considerable positive effect in both DPPH and ORAC values with reference to unsupplemented milk. The mean values of DPPH and ORAC were increased ($p < 0.05$) from 75.44 mg GAE/g and 355.14 µmol TE/g respectively, in the sample after the fermentation until the 21$^{th}$ day of cold storage when their level start to decrease during the last week. This result may be related to the solubilization of reducing substances in the fermented milk. High and significant positive correlation was found in carob fermented milk between TPC and both DPPH radical scavenging activity and ORAC ($R^2 = 0.98$; data not shown). This signifies that phenolic compounds are good radical scavengers. The high content of polyphenols present in carob is most certainly responsible for the antioxidant capacity exhibited in carob fermented milk. These results corroborate with the study of Munian(Muniandy*et al.*, 2016) who reported an increase in DPPH values of yogurts supplemented with aqueous extracts of green, black and white tea (*Camellia sinensis*) stored for 21 days. Similarly, (Ramos *et al.*, 2017) found that the incorporation of polyphenol-rich extract containing 87.5% cloves and 12.5% green mate increase the antioxidant activity in fermented milks.

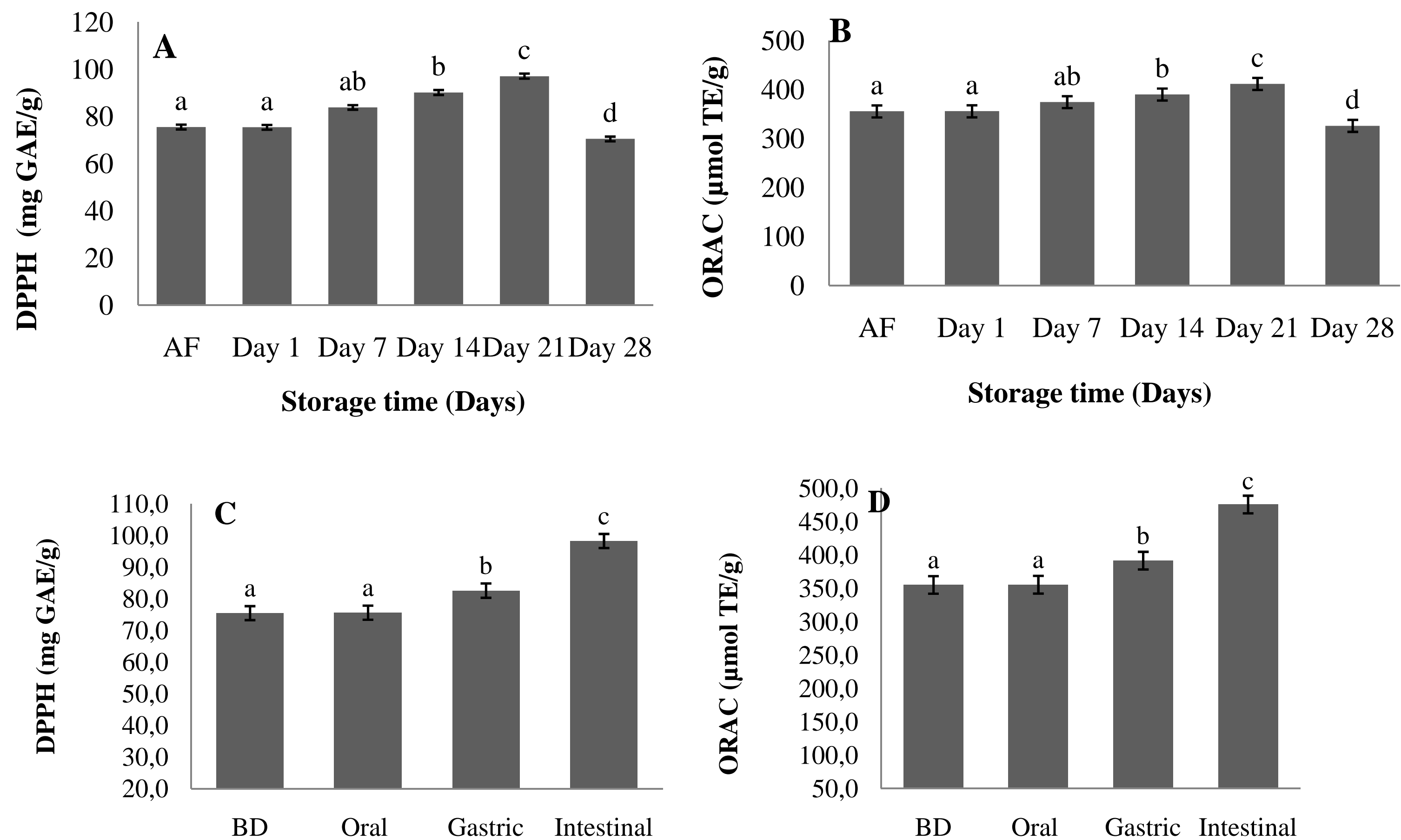


**Fig 2.** Antioxidant activity (DPPH and ORAC) of carob fermented milk during the refrigerated storage (4°C) (A, B) and during the *in vitro* digestion (C, D). Small letters indicate significant differences ($p < 0.05$) during storage period and different steps of digestion of the fermented milk. AF: after fermentation (16 h), BD: before digestion.

## 2.4 Carob fermented milk during the gastrointestinal digestion: TPC, phenolic profile and antioxidant capacity

Gastrointestinal digestion is of major importance to assess the bioaccessibility of polyphenols and their amount released from the food matrix. Thus, in order to understand the effect of the digestion process, the fermented milk supplemented with carob (FCP12) and none supplemented one (control) were submitted to an *in vitro* simulation model of human digestion.

**TPC:** Changes in TPC of carob fermented milk at different digestion stages are set out in Fig 1 (B). The total phenolic content presented no significant ($P > 0.05$) changes in carob fermented milk during the salivary phase compared to the initial content (before digestion). After the gastric digestion, a slight increase ($p > 0.05$) in TPC was observed. A further, the intestinal step had more effect and increased significantly ($P < 0.05$) the total content to 7.04 mg GAE/g of fermented milk leading to a bioaccessibility of 128.47%. The same trend has been previously described in stirred cinnamon-fortified yogurt in which the TPC content reached 86.7% after gastrointestinal digestion (Helal & Tagliazucchi, 2018). The increment of TPC during simulated digestion could be the result of digestive enzymes action and pH changes on polyphenols (conjugated and bounds) facilitating their hydrolysis and their gradual release into the digestive juice (Zhang *et al.*, 2017). Helal & Tagliazucchi (2018) explained also this increase by the protective effect of the milk matrix, which bind the phenolic compounds and make them no longer available for the interaction with pepsin; and it's only when the digestion proceeds, milk proteins are hydrolysed and polyphenols can be released from milk proteins resulting in an increased of bioaccessibility.

**Polyphenolics profile:** Table 5 shows the evolution and the bioaccessibility of individual phenolic compounds of carob fermented milk during the *in vitro* digestion. At the end of the digestion process, the amount of phenolic acids and flavonoids were significantly increased ($P < 0.05$) by 3-fold compared to that before digestion. As can be observed, all the individual phenolic compounds identified in carob fermented milk were bioaccessible but showed different behavior during the digestion. The greatest bioaccessibility was marked by gallic acid (441.74%) followed by chlorogenic acid (305.19%). However, ferulic acid showed the highest loss with a bioaccessibility index of 136.94% after the three steps of digestion.

**Table 5.** Phenolic compounds of carob fermented milk (FCP12) during *in vitro* digestion*.

| Phenolic compounds | Carob powder | BF | Oral | Gastric | Intestinal | Bioaccessibility (%) |
|---|---|---|---|---|---|---|
| Gallic acid | 162.50 ± 0.12 | 102.35 ± 0.14[a] | 104.56 ± 1.02[a] | 234.04 ± 1.03[b] | 452.12 ± 0.99[c] | 441.74 |
| Proto-catechuic acid | 39.19 ± 0.04 | 24.71 ± 0.04[a] | 24.53 ± 1.02[a] | 44.76 ± 0.99[b] | 65.49 ± 1.05[c] | 265.03 |
| Chlorogenic acid | 54.12 ± 0.11 | 33.67 ± 0.24[a] | 36.78 ± 0.05[a] | 78.23 ± 1.02[b] | 102.76 ± 0.75[c] | 305.19 |
| Caffeic acid | Nd | Nd | Nd | Nd | 6.45 ± 0.08[a] | Nd |
| p-coumaric acid | 12.77 ± 0.11 | 8.11 ± 0.18[a] | 10.02 ± 0.08[a] | 16.06 ± 0.45[a] | 21.12 ± 0.98[a] | 260.41 |
| Ferulic acid | 13.44 ± 0.12 | 7.47 ± 0.14[a] | 8.45 ± 0.99[a] | 15.17 ± 0.87[b] | 10.23 ± 0.07[a] | 136.94 |
| o-coumaric acid | 11.07 ± 0.04 | 6.33 ± 0.20[a] | 5.33 ± 0.05[a] | Nd | Nd | Nd |
| *Trans*- cinnamic acid | 42.40 ± 0.15 | 24.10 ± 0.11[a] | 26.12 ± 0.08[a] | 56.58 ± 0.99[b] | 67.44 ± 1.05[b] | 279.83 |
| **Total phenolic acids (TPA)** | **335.46 ± 0.21** | **206.74 ± 0.15[a]** | **215.79 ± 0.17[a]** | **444.84 ± 0.21[b]** | **725.61 ± 0.24[c]** | Nd |
| (+)-Catechin | 11.02 ± 0.03 | 8.23 ± 0.10[a] | 7.49 ± 0.08[a] | 26.19 ± 0.08[b] | 40.02 ± 1.05[c] | 486.26 |
| Rutin | 118.36 ± 0.02 | 88.76 ± 0.15[a] | 90.02 ± 0.10[a] | 128.73 ± 0.88[b] | 264.14 ± 1.10[c] | 297.58 |
| Myricetin | 18.17 ± 0.04 | 10.53 ± 0.11[a] | 12.37 ± 0.08[a] | 28.21 ± 0.09[b] | 35.12 ± 1.00[b] | 333.52 |
| Isoquercitrin | 24.47 ± 0.06 | 15.20 ± 0.14[a] | 16.75 ± 0.66[a] | 8.12 ± 0.06[b] | 5.36 ± 0.09[b] | 35.26 |
| **Total flavonoids (TF)** | **172.02 ± 0.14** | **122.72 ± 0.10[a]** | **126.63 ± 0.11[a]** | **191.25 ± 0.16[b]** | **344.64 ± 0.14[c]** | Nd |
| **Total phenolics (TPA+TF)** | **510.88 ± 0.23** | **329.46 ± 0.17[a]** | **342.42 ± 0.16[a]** | **636.09 ± 0.20[b]** | **1070.25 ± 0.25[c]** | Nd |

*Results are expressed as μg of individual compound in 1 g of carob fermented milk.Means followed by the different letters (a,b) in the same row represent a statistically significant difference ($p < 0.05$). **BF:** Before digestion. **Nd:** not detected/determined

Concerning the flavonoids content, the higher bioaccessibility was observed in (+)-catechin and myricetin with 486.26 % and 333.52 %, respectively. Isoquercitrin was the lowest bioaccessible flavonoid (35.26%). During the digestion, free phenolic compounds were released from the food matrix resulting in increasing their bioaccessibility index. Previous studies reported that the presence of dairy matrices significantly improved the amounts and the stability of polyphenols during the digestion, as the interaction between polyphenols and milk proteins exhibited a protective effect (Green*et al.*, 2007).

**Antioxidant capacity:** Changes in the antioxidant capacity were also investigated in carob fermented milk during the digestion, and the data are depicted in Fig 2 (C, D). Considering the changes found in TPC described above, a similar trend was observed in the antioxidant effect of carob fermented milk submitted to *in vitro* digestion. During the passage throughout the simulated digestion system, the DPPH and ORAC values of fortified carob fermented milk increased progressively to 98.23 mg GAE/g and 475.61 µmol TE/g respectively being 130% and 134% higher than the initial values. These results are in accordance with the data reported by (Oliveira & Pintado, 2015) for the *in vitro* digestion of strawberry and peach yoghurt.

## 2.5 Cell viability of probiotics in carob fermented milk under the *in vitro* gastrointestinal simulation

The ability to survive the digestive stresses and reach the intestine in large numbers is one of the fundamental properties of probiotics that can be successfully incorporated into food. Fig 3 illustrates the changes in cell viability of the probiotic strains in carob fermented milk and MRS broth (control) during the gastrointestinal simulation. The gastric conditions reduced significantly ($p < 0.05$) the population of the probiotic lactobacilli by1 log. The effect of intestinal step was more accentuated and slowly decreased ($p > 0.05$) the number of lactobacilli to 7.22 log CFU/mL. This reduction in probiotic number in the fermented milk under gastrointestinal conditions was also reported by previous works (Casarotti & Penna, 2015; Moreno-Montoro *et al.*, 2018). As expected, the lactobacilli (*Lb. brevis* B13 and B38) strains survived better when incorporated in carob fermented milk compared to the MRS broth. Overall, the viability of the used bacteria was maintained at an acceptable concentration and exceeded the minimum required to confer health benefits (6 log CFU/mL). The carob powder improved the lactobacilli viability and tolerance to the harsh conditions of gastrointestinal tract. In addition,

the food matrix and its components may create an environment that confers protective effects to probiotic strains during the passage throughout the gastrointestinal tract. Moreover, food components could bind to bile acids, reducing their toxic effect on probiotic cells (Begley*et al.*, 2005).

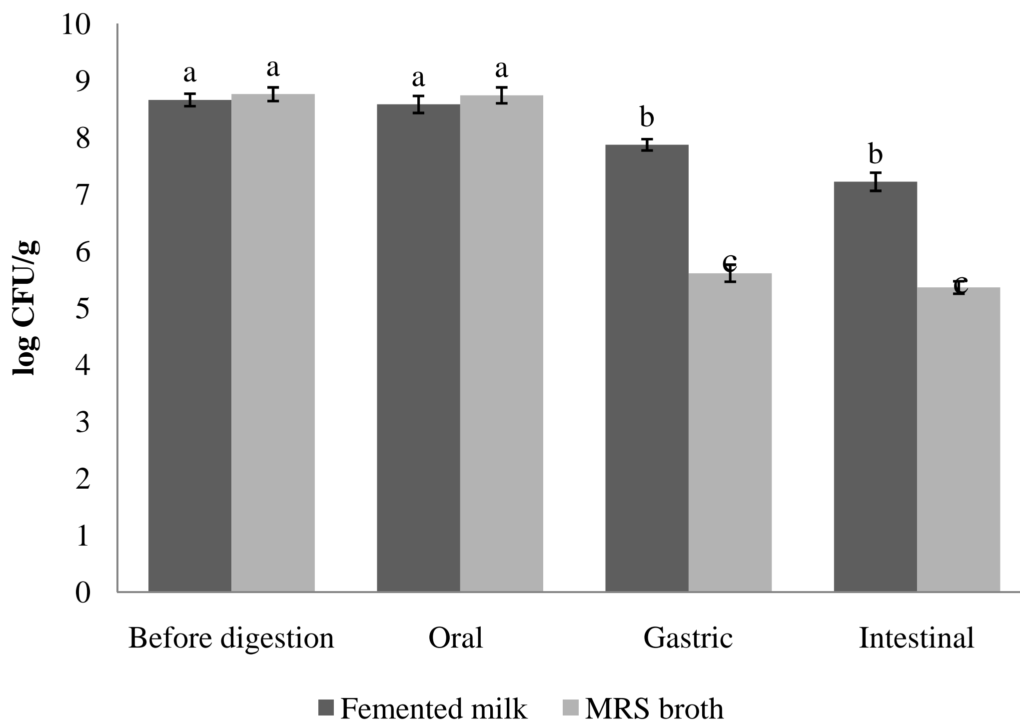


**Fig 3.** Survival of both probiotic strain (*Lb. brevis* B13 and B38) in carob fermented milk (FCP12) during the simulated gastrointestinal digestion. Small letters indicate a statistically significant difference between before the *in vitro* digestion counts and counts at each digestion step.

## 2.6 α -Amylase and α -glucosidase inhibition

*In vitro* simulated digestion of carob fermented milk made a significant improvement on α-amylase and α-glucosidase inhibitory activity Fig. 4 (A, B).

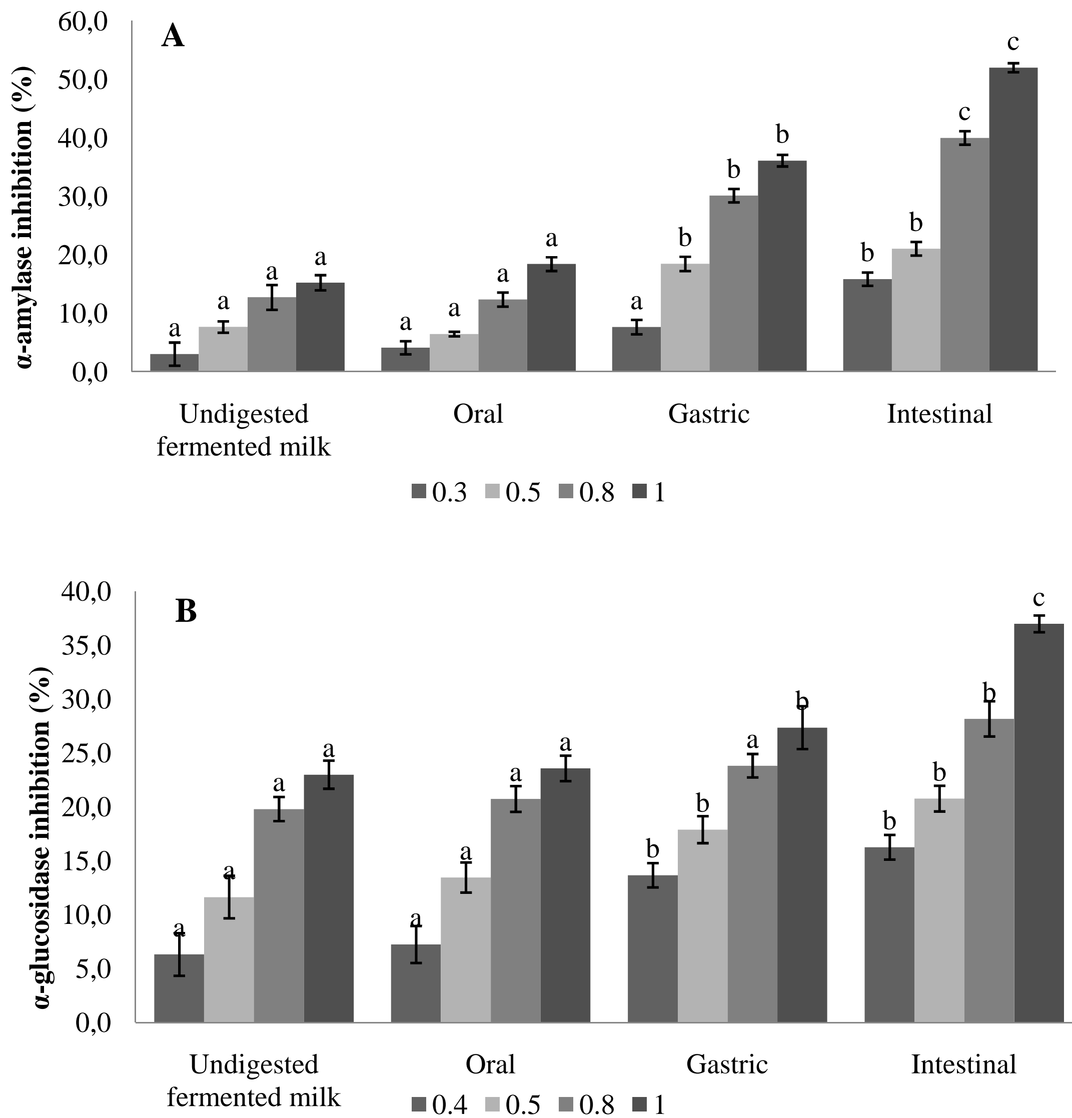


**Fig 4.** α-amylase (A) and α-glucosidase (B) inhibition (%) of carob fermented milk (FCP12) and its digested fractions. Small letters indicate significant differences before and during digestion steps for each tested concentration (0.3, 0.4, 0.5, 0.8 and 1 mg/mL).

It could be seen that the inhibitory effect of α-amylase and α-glucosidase in digested fractions was more effective than before the digestion and increased progressively ($P < 0.05$) up to 3.4 and 1.6-fold respectively. As mentioned above, the intestinal fractions exhibited high total phenolics content suggesting that the inhibition of both enzymes is dependent on polyphenolic compounds amounts. In addition, the inhibition of the α-amylase and α-glucosidase enzymes could be attributed also to bioactive peptides generated by the proteolytic activity of the probiotic strains (Gomes da Cruz*et al.*, 2009). The inhibition of α-amylase and α-glucosidase activities can be considered as an effective approach to controlling diabetes by reducing carbohydrate metabolism (Donkor*et al.*, 2012). The findings from this study show that the fermented milk enriched with carob powder had the potential to manage post-prandial hyperglycemia.

## 3 Conclusion

The results of the present work confirm that the addition of carob powder into fermented milk improve the probiotic (*Lb. brevis* B13 and B38) growth during the fermentation process and maintained their viability during the entire storage period (28 days). The carob stimulates also the acidifying activity of the used culture and increases the viscosity of fermented milk. The data showed an increment of the total phenolic content and the antioxidant capacity during storage in carob fermented milk. Carob powder has proven to be an alternative ingredient in fermented milk formulation, contributing to the development of a functional food and a synbiotic product, with high bioaccessibility polyphenols after the simulated gastrointestinal digestion, enhanced antioxidant properties, large viable numbers of probiotics and significant hypoglycemia activity. Further studies need to be performed to examine the consumer sensory evaluation of the fermented milk to elucidate the most important sensory descriptors and the flavor compounds.

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## Chapter 4. General discussion

### 1 Overview and significane

The work conducted in this thesis examined the feasibility of developing novel synbiotic fermented milks in Algeria using local starters and adjunct cultures (probiotics) and raw product (carob powder) as a prebiotic ingredient in attempts to valorize the local sources.

The ***first experimental part*** of this study aimed to isolate and identify autochthonous lactic acid bacteria (LAB) strains (lactococci and lactobacilli) from Algerian homemade cheeses to be used later in the development of the fermented milk as starter and probiotic cultures respectively.

A total of twelve (12) isolates were found to be lactococci strains based on their morphological and physiological properties. The selection and screening of these lactococci were based on their antibacterial activity toward two foodborne pathogens (*E. coli* and *S. aureus*), used as representative of Gram-negative and Gram-positive bacteria. Most of the isolated lactococci were active against one or both target strains, but the strain C15 expressed a powerful antibacterial activity against the both target strains with inhibition zone diameter reaching 15 mm. This strain was later identified genotypically with 16S rDNA sequencing as *Lactococcus lactis*. In general, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* are mostly found in dairy products, including raw milk, soft and hard cheeses, and sour cream (Casalta & Montel, 2008). The antimicrobial activity of starter cultures is important since it contributes to the inhibition of the development of pathogenic and food-degrading microorganisms that are hamfull for the consumer and shorten the shelf-life of food products. When we examined the biochemical and technological properties of the *Lc. lactis* C15, this latter exhibited an interesting capacity to acidify the milk, good antioxidant activity, high lipolytic and proteolytic activities which are desired properties in dairy foods for development of aroma and texture.

In another hand, a total of 98 lactobacilli isolates were obtained from the homemade cheeses and subjected to probiotic potentialities study. To select the good candidates, these isolates were firstly screened based on their antibacterial activity against *E. coli* and *S. aureus*. In fact, one of the most important aspects of probiotics is antagonistic activity against pathogenic

microorganisms. The antagonistic potential of lactobacilli could be related to different factors, such as reduction of the pH values, competition for substrates, and production of bacteriocins or low-molecular-weight peptides (Avci *et al*., 2015, Viana de Souza & Silva Dias, 2017). Fourteen (14) lactobacilli isolates showed a pronounced antibactertial activity and were selected for further characterization. From these, two lactobacilli isolates (B13 and B38) identified by 16S rDNA sequencing as *Lactobacillus brevis* showed significantly better results in trems of survival under gastrointestinal digestion, adhesion ability, cholesterol lowering, antioxidant activity and technological properties.

The findings from this first part of our project showed that *Lc. lactis* C15 and both *Lb. brevis* B13 and B38 possess interesting functional traits and could be good candidates for industrial use as starter and probiotic cultures. The application of these bacteria in fermented dairy products will contribute to enhance their quality, with increased added value and functional properties. We can conclude also from these results that the Algerian artisanal fermented foods and especially the dairy products could be a potential source and rich reservoir to search for interesting cultures that can be used in development of fermented foods to replace the imported commercial cultures. Unfortunately, in Algeria, the microbial community of fermented foods is not well studied or even exploited for industrial interest; except some few works related to the isolation of some functional strains from fermented dairy products such as Jben, Klila, Raib and Lben for application in fermentation (Mechai *et al.*, 2014; Bachtarzi *et al.*, 2019).

In the ***second section*** of this project, we focused our investigations on the study of the composition of carob powder in terms of polyphenols and dietary fibers and its potential as a prebiotic ingredient. The carob is known in the Mediterranean region for its importance and usefulness especially its raw pods which have been progressively employed in various industries during the past few years (Gubbuk *et al.*, 2010).

As mentioned above in the chapter 1, an ideal prebiotic should be (1) resistant to the deleterious action of the gastrointestinal digestion (2) should not be absorbed in the upper gastrointestinal tract, (3) should be easily fermentable by the beneficial intestinal microflora (Kuo, 2013). So, the carob powder was submitted to an *in vitro* gastrointestinal digestion and a fecal microbial fermentation.

The analysis of the polyphenol's composition of the carob before the digestion revealed a high content of phenolic acids and flavonoids present in conjugated and bound forms. Under the gastrointestinal digestion (low pH and digestive enzymes), the bound polyphenols were degraded, and the free forms were released. So, the carob polyphenols were stable after digestion and became more accessible, resulting in higher biological activities (antioxidant activity, α-amylase and α-glucosidase inhibition). The results showed also that after digestion, there is an amount of polyphenols that remains non digested, that can be fermented an metabolized by the gut microbiota resulting in production of metabolites with health benefits.

These findings show clearly the efficacy of carob powder to be a functional prebiotic that can be successfully employed in the dairy fermentation. This project offers new insights to better increase the use of carob in Algeria as the Algerian industry did not give attention to this rich product.

Using the starter (*Lc. lactis* C15) and both probiotic *(Lb.brevis* B13 and B38) cultures previously characterized with the addition of 4% (w/v) of carob powder, we successfully developed a novel synbiotic fermented milk as reported in the ***third section*** of this thesis.

Considerable interactions between *Lc. lactis* and probiotic strains took place during milk fermentation and refrigerated storage of fermented milk. The acidification kinetic parameters (pH and Tirable Acidity) of fermented milk and its viscosity were influenced by the addition of carob powder, and the best kinetic parameters were obtained for the milk fermented with *Lc. lactis* C15 and the co-culture of *Lb. brevis* B13 and B38. The carob fermented milk displayed higher polyphenolics content, higher antioxidant capacity and maintained the viability of the probiotic culture (>$10^7$ CFU/g) over fermentation and storage. Obtaining desirable therapeutic effects in probiotic fermented milks requires maintaining of the probiotic cultures viability at a sufficient level throughout storage of the product. It has been suggested that probiotics should be present in the food product in minimal amounts of $10^6$ CFU/g. This amount could be translated into $\geq 10^6$ CFU/g/day of probiotics containing fermented milk given that 100 g is the daily serving portion. Such high dosage is required to compensate the loss of cells during the passage through the upper and lower parts of the gastrointestinal tract (Granato *et al.*, 2010). From a technological standpoint, fermented milk supplementation with probiotic cultures is not easy to developpe, particularly with respect to maintaining the viability of the cultures. Many factors

influence the viability of probiotics in fermented milk: strain variation, acid accumulation, interaction with starter cultures, levels of dissolved oxygen and hydrogen peroxide ($H_2O_2$), and storage conditions (Donkor *et al.*, 2006). Several studies reported that some commercially available dairy products contain insufficient number of viable probiotics (as defined by $>10^6$ CFU/g or mL before the expiration date), thereby diminishing the potential health benefits conferred by these products (Lin *et al.*, 2006).

When the carob fermented milk was submitted to the gastrointestinal digestion, probiotic cultures maintained also the viable count ($>10^7$ CFU/g) higher than the minimum level recommended for the beneficial health properties in the gut. Indeed, the supplementation of the fermented milk with this prebiotic improved *Lactobacillus brevis* resistance to the simulated gastrointestinal conditions. The protective effect of prebiotics on probiotic cultures during the simulated digestion was widely observed in previous studies with other ingredients (Agil *et al.*, 2013; Li *et al.*, 2016; Batista *et al.*, 2017). The incorporation of fruit flour into milk can be an interesting alternative for functional fermented milk production because it may lead to higher appeal of the product to consumers as well as to increase the viability of probiotic strains in the gut.

The *in vitro* digestion of carob fermented milk resulted in the release of phenolic compounds from milk proteins so that at the end of the digestion the amount of phenolic compounds was higher than before digestion. As a result, the antioxidant capacity of the digested milk was increased as well as its hypoglycemia activity (inhibition of α-amylase and α-glucosidase). These results clearly showed that fermented milk matrix enhance the gastro-intestinal stability and the bioaccessibility of carob polyphenols.

In summary, we can conclude that the carob fermented milk developed in this project can be considered as an important source of viable functional probiotics and a source of dietary bioaccessible polyphenols that may confer health benefits to consumers.

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## General conclusions and perspectives

Due to their benefits andthe increasing importance of healthy lifestyle, functional foods, among them fermented milks, are becoming a part of consumers' everyday live. In Algeria, the consumption of fermented dairy products is a part of the daily diet and people are demanding more innovative products to preserve their health. In this sense, it is time to focus on developing novel products using local sources in a way to enrich the Algerian dairy industry.

Our investigation provides the first evidence in Algeria and proposes a strategy to successfully obtain a potential, functional and synbiotic fermented milk using newly isolated probiotic bacteria and carob powder as a prebiotic ingredient.

One hundred and ten (110) bacterial strains (98 lactobacilli and 12 lactococci) were isolated and firstly screened based on their antibacterial activity towards two selected potential harmful pathogens (*Escherichia coli* and *Staphylococcus aureus*) that are considered as food contaminants. Then, among the lactococci group, one *Lactococcus lactis* strain showing a good technological trait (acidifying activity) was selected to be used as a starter culture to manufacture the fermented milk. Parallalely, two *Lactobacillus brevis* strains (B13 and B38) with interesting and promising probiotic potential (good *in vitro* adherence to human HT-29 cell andtolerance to low pH, bile and pancreatic fluid) were selected. These results clearly indicate that the Algerian artisanal cheeses could be a potential source of starter and adjunct cultures, which can be used in dairy products manufacture in Algeria.

The analysis of the carob polyphenols composition revealed a good amount of free, conjugated and bound fractions, which were gradually liberated during the gastrointestinal digestion of the carob powder resulting in an increase of the antioxidant capacity and the ability to inhibit enzymes involved in carbohydrate metabolism.

Thus, the carob powder was successfully incorporated in the fermented milk to enhance its functionality. Indeed, adding the carob powder at 4% maintained the viability of both probiotic *Lactobacillus brevis* strains during the fermentation process, the storage at 4°C/28 days and during the *in vitro* gastrointestinal digestion of the fermented milk ($\geq 10^7$ CFU/mL).

In addition, the carob fermented milk showed high polyphenols content with high bioaccessibility after the simulated gastrointestinal digestion as well as high antioxidant properties and outstanding bioactivities. These results prove that carob powder can be used as a prebiotic ingredient in fermented milk formulation to improve the growth of probiotic bacteria and it should be recommended as a novel ingredient to enhance fermented milk bioactivities.

Overall, the finding gathered from this work may be of great interest to the dairy industry speeding up the initial steps for companies to launch into the Algerian market distinguishable functional products with more aggregated value using local raw products.

For the future, further studies should focus on:

- Performing the sensory profiling (flavor compounds) of the carob fermented milk with a trained panel using descriptive analysis and compare it to other commercial fermented milks available in the Algerian market to explore the consumer acceptability of this product.
- Study the health benefits and bioactivities of the carob fermented milk *in vivo* in a clinical study considering animal model or humans.
- Illuminating the interaction between the phenolic composition of carob powder and the enzymatic profiles of the probiotic bacteria.

**Appendix**

**Table 1.** Antimicrobial activity of cell free supernatants (CFSs) of somelactococci / lactobacilli strains against *S.* enterica and *L. monocytogenes* (mm)

| Strains | Inhibition zone (mm) | | | | |
|---|---|---|---|---|---|
| | CFSs | | | Neutralized CFSs (pH=6.5) | |
| | pH | *Sal. enterica* | *L. monocytogenes* | *Sal. enterica* | *L. monocytogenes* |
| C15 | 3.18[a] | 17± 1.02[bc] | 13± 0.44[bc] | 0 | 0 |
| C9 | 3.69[a] | 10 ± 0.66[e] | 12 ± 0.52[cde] | 0 | 0 |
| B9 | 3.80[cd] | 9 ± 0.98[cde] | 10 ± 1.14[def] | 0 | 0 |
| B13 | 3.83[d] | 12 ± 1.02[bc] | 12 ± 0.35[f] | 0 | 0 |
| B38 | 3.83[d] | 10 ± 0.26[a] | 9 ± 0.22[def] | 0 | 0 |

**a) Supplementary figures**

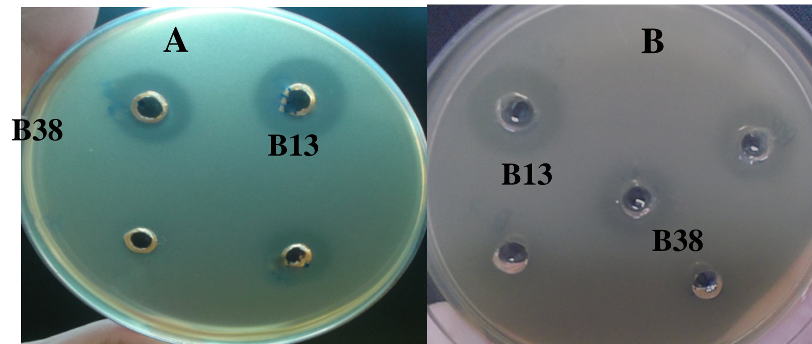


**Fig. 1:** Inhibition zone of CFS of *Lb. brevis* B13 and B38 agaisnt *E. coli* (A) and *S. aureus* (B)

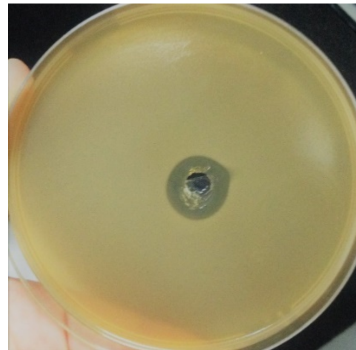

**Fig. 2:** Inhibition zone of neutralized CFS of *Lc. lactis* (C15) against *S. aureus*

**Fig. 3:** Carob milk fermented with *Lc. lactis* (starter) and supplemented with *Lb. brevis* B13 and B38 (probiotic).

b) **Phenolic chromatogramsof carob powder before and after gastrointestinal digestion**

**Free phenolics**

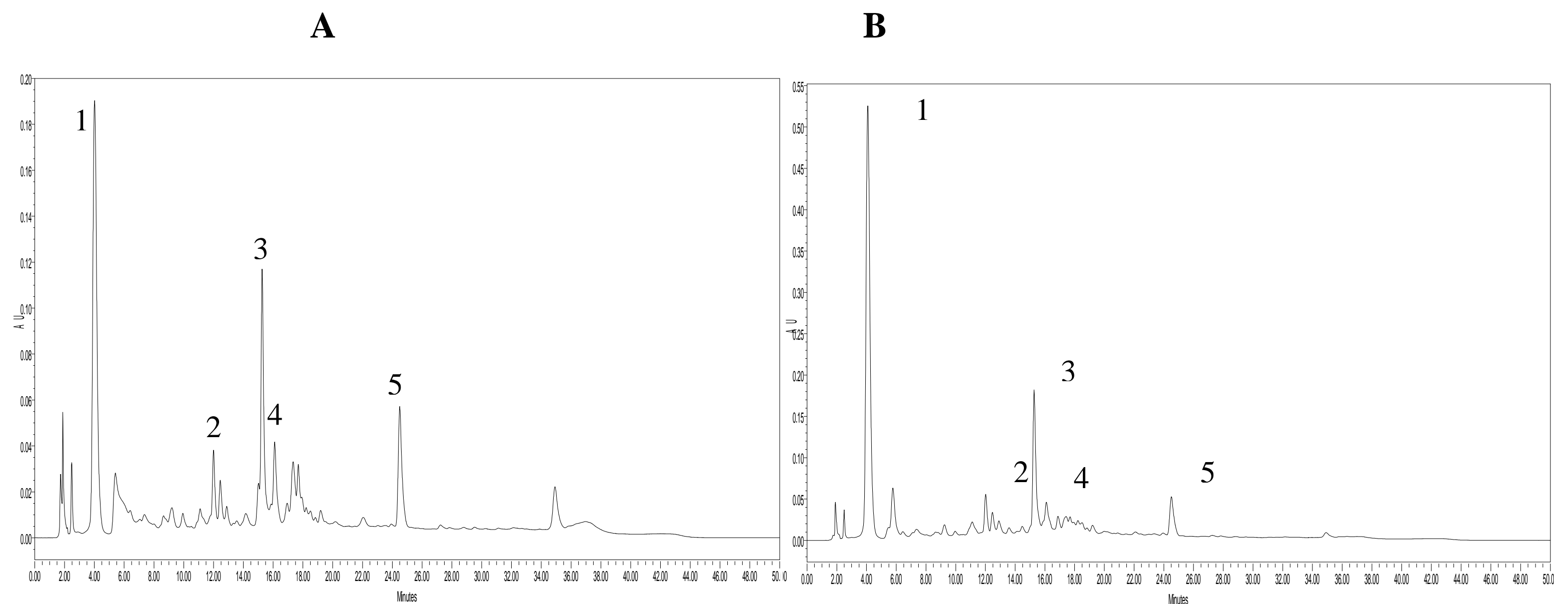


**Fig. 4.**HPLC-DAD chromatograms of soluble freein non-digested (A) and digested (B) carob samples during simulated *in vitro* digestion at 280 nm. Peaks are: (1) Gallic acid, (2) proto-catechuic acid, (3) Chlorogenic acid, (4) Ferulic acid, (5) *Trans*- cinnamic acid.

## Insoluble bound phenolics

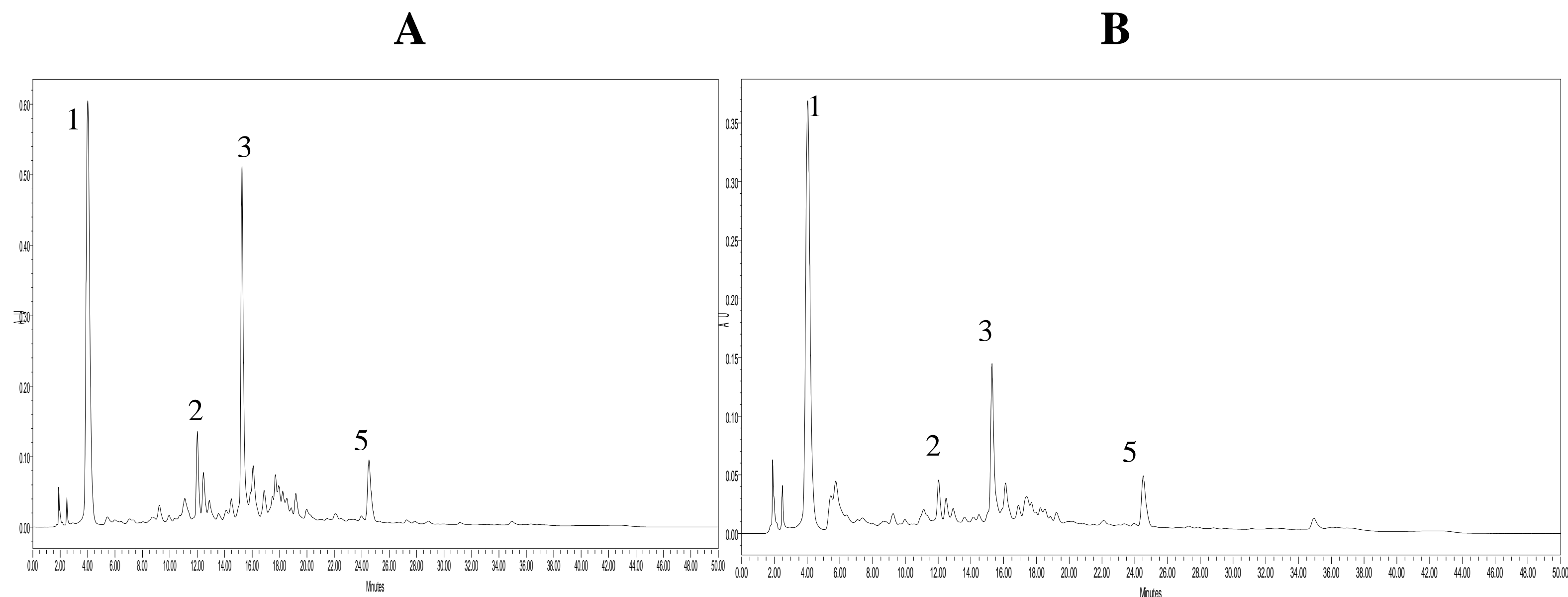


**Fig. 5:**HPLC-DAD chromatograms of insoluble bound phenolics in non-digested (A) and digested (B) carob samples during simulated *in vitro* digestion at 280 nm. Peaks are: (1) Gallic acid, (2) proto-catechuic acid, (3) Chlorogenic acid, (4) Ferulic acid, (5) *Trans*-cinnamic acid.

## Free flavonoids

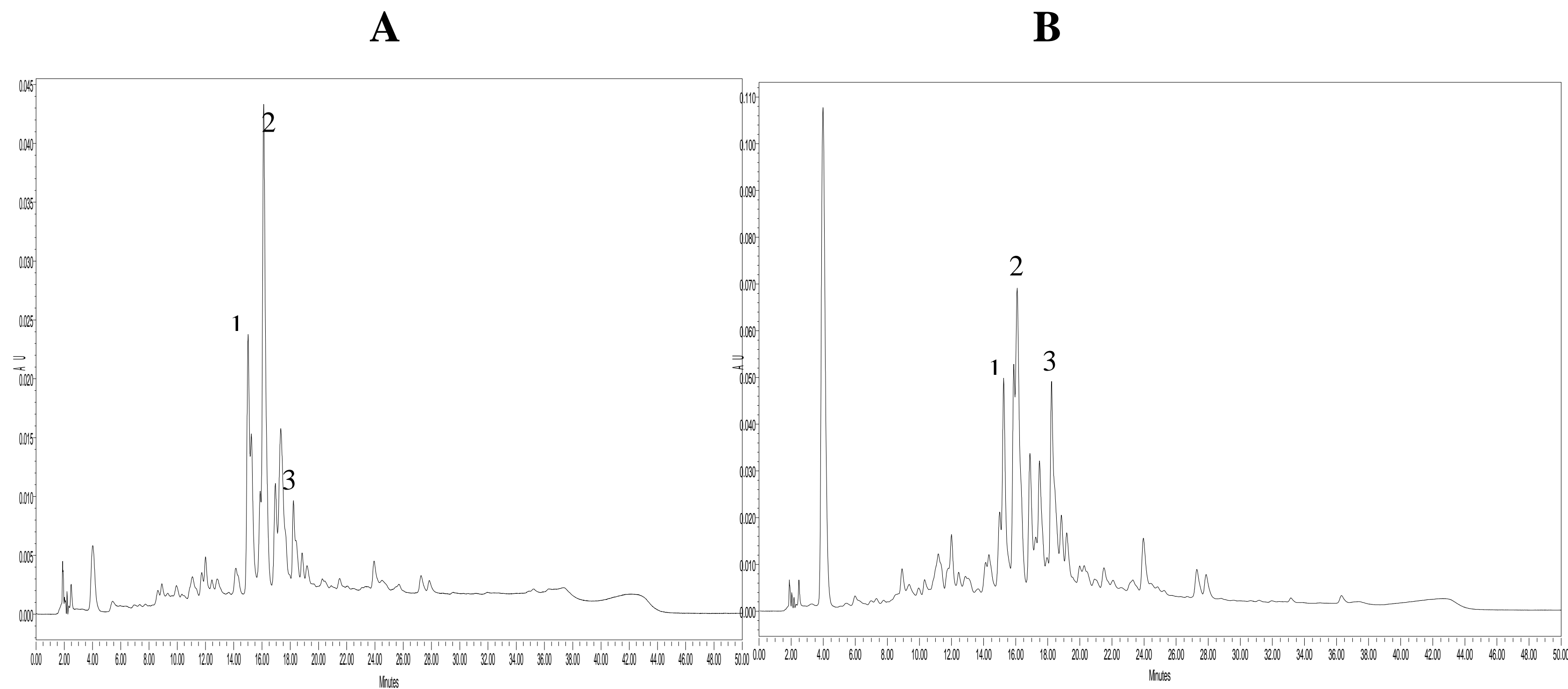


**Fig. 6:**HPLC-DAD chromatograms of soluble free flavonoids in non-digested (A) and digested (B) carob samples during simulated *in vitro* digestion at 320 nm. Peaks are: (1) (+)-catechin, (2) rutin, (3) myricetin, (4) isoquercitin.

## Insoluble bound flavonoids

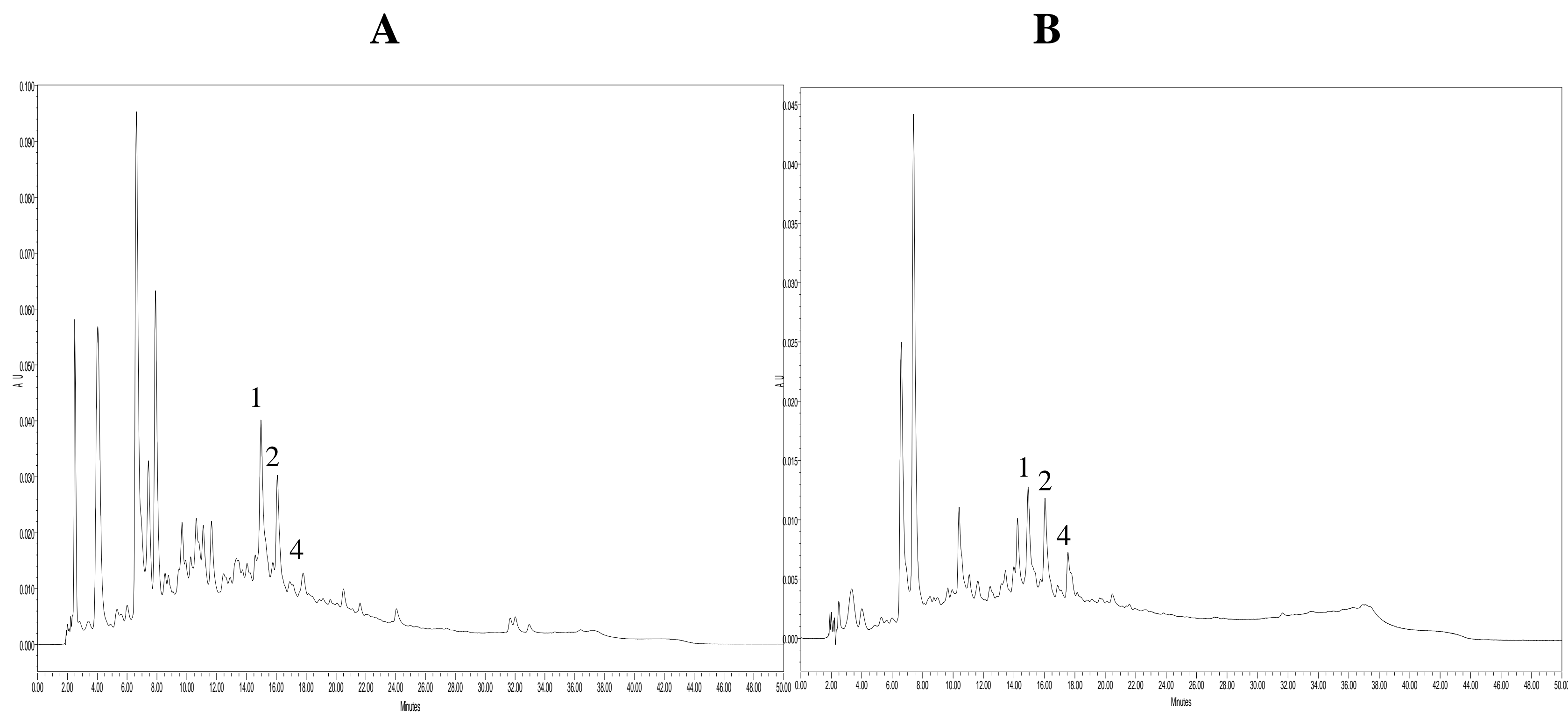


**Fig. 7:** HPLC-DAD chromatograms of insoluble bound flavonoids in non-digested (A) and digested (B) carob samples during simulated *in vitro* digestion at 320 nm. Peaks are: (1) (+)-catechin, (2) rutin, (3) myricetin, (4) isoquercitin.

## c) Published paper

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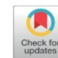
# Simulated gastrointestinal digestion and *in vitro* colonic fermentation of carob polyphenols: Bioaccessibility and bioactivity



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Phenolic compounds

ABSTRACT

Carob is a well-known tree for its nutritional and health-promoting edible pods due to its high phenolic contents. It was aimed to investigate: 1) carob phenolic profiles; *soluble free, soluble conjugated and bound,* 2) bioaccessibility of those phenolics during simulated gastrointestinal digestions, plus *in vitro* fecal fermentations, 3) their antioxidant properties, also 4) α-amylase and α-glucosidase inhibitory activities. Ten phenolic acids and six flavonoids were detected in soluble (free and conjugated) and insoluble fractions of undigested carob. After *in vitro* gastrointestinal digestions, the most bioaccessible phenolic acids and flavonoids were gallic acid (647.4%), chlorogenic acid (485.4%), (+)-catechin (558.3%) and rutin (267.2%). Myricetin (79.5%) and gallic acid (20.0%) were the most abundant metabolites of residual fraction of carob phenolics after fecal fermentations. Antioxidant capacity of digested carob has increased significantly ($p < 0.05$); DPPH (107 mg GAE/g), ABTS (399 mg TE/g) and ORAC (415 μmol TE/g). Both undigested carob and its digested fractions showed a positive dose-dependent inhibition of α-amylase activity (0.3–1.0 mg/mL). The findings from this study showed *first time* report on carob phenolic profiles (soluble and insoluble) and their bioaccessibility during digestions and fecal fermentations. Carob phenolic might influence glucose metabolism by inhibiting carbohydrate digestion.

## 1. Introduction

Carob (*Ceratonia silique* L.) is an evergreen tree cultivated or naturally grown in the Mediterranean basin (Dakia, Wathelet, & Paquot, 2007; Yousif & Alghzawi, 2000). Algeria is considered one of the most carob producing countries with a yield of approximately 3000 tons per year (Nasar-Abbas et al., 2016). Carob fruits have been widely used in food, pharmaceutical and cosmetic industries. The most reported health benefits of carob are cholesterol lowering effects (Ruiz-Roso, Quintela, de la Fuente, Haya, & Pérez-Olleros, 2010), blood glucose level regulations (Bañuls et al., 2016; Gruendel et al., 2007), antimicrobial effects and high antioxidant potentials (Gruendel et al., 2006; Klenow, Jahns, Pool-Zobel, & Glei, 2009; Kumazawa et al., 2002). Also, other few studies have reported *in vitro* anticancer and anti-proliferative effects on mouse hepatocellular carcinoma (Corsi et al., 2002), HT29 colon adenocarcinoma cells and LT97 colon adenoma cells (Klenow, Glei, Haber, Owen, & Pool-Zobel, 2008). The biological activities of carob pods are mainly attributed to their high polyphenolic content especially condensed tannins (Ayaz et al., 2009).

Phenolic compounds are the major complex groups of phytochemicals that may exist in plant cells as soluble free, soluble conjugated (also known as esterified phenolics) and insoluble-bound forms (Jung, Jeon, & Bock, 2002; Madhujith & Shahidi, 2009; Žilić et al., 2011). Typically, soluble conjugates and bound phenolics can be released by acidic, alkaline or enzymatic hydrolysis method (Kim, Tsao, Yang, & Cui, 2006). Studies demonstrated that phenolic conjugates and bound ones play an essential role as antioxidants (Pérez-Jiménez & Saura-Calixto, 2015). Most of research conducted on carob phenolic shave focused on soluble free forms (Benchikh, Louaileche, George, & Merlin, 2014; Bernardo-Gil et al., 2011; Roseiro et al., 2013) although the studies of conjugated and bound compounds have been started since 1980s (Krygier, Sosulski, & Hogge, 1982; Naczk & Shahidi, 1989).

To our knowledge, there is very limited information about carob phenolic profiles in soluble and non-soluble fractions. Therefore, for better understanding the potential health benefits of carob consumption, it is crucial to investigate soluble free, soluble conjugated and bound phenolics and their antioxidant activity. Additionally, the health benefits of bioactive polyphenols depends on their bioaccessibility and the way in which they are metabolized in human body (Rein et al., 2013). Thus, it is important to evaluate their stability and absorption in

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the digestive tract. *In vitro* digestion models are a powerful approach simulating *in vivo* conditions to predict changes in stability and bioavailability of compounds in food plants (Bohn et al., 2017; Thomas-Valdés, Theoduloz, Jiménez-Aspee, Burgos-Edwards, & Schmeda-Hirschmann, 2018). No study has yet investigated the effects of *in vitro* digestions on carob conjugates and bound phenolics and their bioactive activities. Very few studies (Ortega, Macià, Romero, Reguant, & Motilva, 2011; Ydjedd et al., 2017) have focused on stability and bioaccessibility of phenolic compounds in carob flour focusing only on free phenolics.

Under the light of these facts, this work was planned to investigate: 1) phenolic profiles of carob in soluble free, soluble conjugate, and insoluble-bound forms, 2) bioaccessibility of those three fractions after simulated gastrointestinal digestion and *in vitro* fecal fermentations, 3) their antioxidant properties, plus their potential α-amylase and α-glucosidase inhibitory activities.

## 2. Material and methods

### 2.1. Chemical and reagents

Human saliva α-amylase (14 μkat/mg proteins), pepsin from porcine gastric (11 μkat/mg), pancreatin from porcin pancreas (4xUPS, 0.12 μkat of trypsin/mg), α-amylase from *Bacillus licheniformis*, α-glucosidase and bile salts were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Solvents including acetone, methanol, diethyl ether, ethyl acetate, acetic acid, acetonitrile and HCl were analytical grade and purchased from Sigma-Aldrich. Folin-ciocalteau reagent, sodium carbonate, aluminium chloride,2, 2-dipheny-1-picryhydrazyl radical (DPPH), fluorescein, trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), 2, 2-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, DNS (3, 5-dinitrosalicylic acid), p-nitrophenyl-α-D-glucopyranoside and NaOH were obtained from Sigma-Aldrich (Oakville, ON, Canada). The phenolic acid standards (over ≥ 980 g/Kg pure); gallic, protocatechic, chlorogenic, caffeic, vanillic, syringic, *p*-coumaric, ferulic, *o*-coumaric, *trans*-cinnamic, the flavanoid standards; (+)-catechin, rutin, isoquercitrin, myricetin, apigenin and kaempferol were also purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

### 2.2. Sample preparation

Ripe carob (*Ceratonia siliqua* L.) pods were collected during July–August 2016 in Bejaia, Algeria. They were cleaned with distilled water and seeds were removed. The pulps were dried in microwave (Hotpoint Ariston, USA) at 720 W for 15 min, ground to a fine powder using a commercial food blender and passed through a 0.149 mm sieve to obtain uniformly sized powder. The samples were stored at − 20 °C until analysis.

### 2.3. In vitro simulated gastrointestinal digestion

The static model proposed by (Minekus et al., 2014) was followed to study the *in-vitro* digestion, including three sequential steps: oral, gastric and intestinal. Stock solutions; *simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)* were prepared as the same molarity as reported in the method. All solutions were daily prepared and pre-warmed at 37 °C before the use. Additionally, salivary α-amylase (mouth phase) was prepared in SSF to a final concentration of 1.25 μkat/mL, pepsin (stomach phase) in SGF to a final concentration of 33 μkat/mL as well as pancreatin (small intestine) in SIF to a final concentration of 2 μkat/mL (based on trypsin activity). ***In the mouth phase***, 8 g (8 g) of carob pulp powder were transferred to 50 mL beaker and mixed thoroughly with 8.4 mL SSF solution, 1.2 mL of salivary α-amylase solution, 60 μL of 0.3 mol/L $CaCl_2$ and 2340 μL of distilled water. Then, the obtained mixture was incubated in a water bath for 2 min at 37 °C. ***Gastric digestion*** was continued by immediate addition of 7.5 mL of SGF, 5 μL of 0.3 mol/L $CaCl_2$, 695 μL of distilled water to the oral bolus and pH was adjusted to 3.0 with enough volume of 6 mol/L HCl. Next, 1.6 mL of porcine pepsin was added, and continuously kept under shaking (120 rpm) at 37 °C for 2 h. Then, ***intestinal digestion*** was followed by the addition of 5.5 mL of SIF, 20 μL of 0.3 mol/L $CaCl_2$ and 1.25 mL of bile salts (25 mg/mL) to the mixture. After adjusting the pH to 7 with 6 mol/L NaOH, 2.5 mL of a pancreatin solution and 655 μL distilled water were added, kept under agitation (120 rpm) at 37 °C for 2 h. Aliquots were collected at the end of each phase (oral, gastric and intestinal) and placed in an ice bath for 10 min to deactivate enzymes (Minekus et al., 2014). Then, samples were freeze-dried (Labconco, Fisher Scientific, USA) and stored at - 20 °C until further analysis.

### 2.4. Phenolic profiles

Soluble free, soluble conjugated and bound phenolics were extracted from digested and non-digested carob according to previously reported method (Krygier et al., 1982), with some modifications. One gram of sample was extracted three times with 20 mL of 800 mL/L acetone at room temperature for 1 h under magnetic stirring. After centrifugation (Sorvall Legend XTR, Thermo Fisher Scientific, Germany) at 4000 g for 10 min, the combined supernatants were analyzed for soluble free and conjugated (esterified) phenolics while the residue was reserved for the determination of insoluble (bound) phenolics. The combined supernatants were evaporated under vacuum using a rotary evaporator (Buchi R-215, Flawil, Switzerland) to remove the acetone and then acidified to pH 2.0.

***Free phenolics*** were extracted three times with diethyl ether-ethyl acetate (1:1, mL/mL). The organic phases were dehydrated with anhydrous sodium sulphate, combined and evaporated to dryness at 30 °C. The aqueous phase obtained after free phenolic extraction was hydrolysed with 20 mL of 2 mol/L NaOH for 4 h at room temperature. The resultant hydrolysate was acidified to pH 2.0 and released ***conjugated (esterified) phenolics*** were extracted with diethyl ether-ethyl acetate as described above. For the ***bound phenolics*** extraction, the solid residue obtained from soluble free phenolics fraction was dissolved in 40 mL of 2 mol/L NaOH whilst stirring for 4 h. The mixture was then adjusted to pH 2.0, centrifuged and the insoluble-bound phenolics were extracted with diethyl ether-ethyl acetate (1:1, mL/mL) in the same manner as explained above. All samples were stored at − 20 °C until analysis.

### 2.5. Total phenolic content (TPC) and total flavonoid content (TFC)

The phenolics in both non-digested and digested samples were further analyzed for their TPC and TFC values as well as their antioxidant capacity. Firstly, phenolic compounds in each sample were extracted with 20 mL of 800 mL/L aqueous acetone containing 10 mL/L acetic acid for 3 h at room temperature. Thereafter, the mixture was centrifuged for 15 min at 4000 g and TPC was spectrophotometrically determined using the modified procedure of the folin-ciocalteu adapted to 96-well plate assay, as described by Gao, Wang, Oomah, and Mazza (2002). The absorbance was read at 725 nm using a microplate reader (Epoch, Biotek, Fisher Scientific, Winooski, USA) and expressed as mg of gallic acid equivalent per g of sample (mg GAE/g). TFC was also determined spectrophotometrically by following the procedure of Zhang et al. (2015), adapted to 96-well microplate (Costar, Corning Incorporated, Conrning, USA) assay. The absorbance was read at 515 nm and results were expressed as mg of Rutin equivalent per g of sample (mg RUE/g) (Zhang et al., 2015).

### 2.6. Antioxidant capacity

#### 2.6.1. DPPH

The antiradical activity of all samples was determined by DPPH (2,2-Diphenyl-1-picrylhydrazyl) (Brand-Williams, Cuvelier, & Berset, 1995). Results were expressed as mg gallic acid equivalent per gram of sample (mg GAE/g) after reading the absorbance at 519 nm after 30 min of incubation.

#### 2.6.2. ORAC

Oxygen radical absorbance capacity (ORAC) values of all samples were calculated using the differences of areas under the fluorescence decay curves between the blank and a sample. The results were expressed as micromole trolox equivalents per gram of sample (μmol TE/g) (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002).

#### 2.6.3. ABTS

The $ABTS^{\bullet+}$ scavenging activity of all samples was conducted as described by Leite et al. (2011). The ABTS•+ solution was produced by reacting aqueous ABTS solution (7 mmol/L) with potassium persulfate (2.45 mmol/L) for 12 h before use. Then, on a 96-well microplate, diluted ABTS•+ solution and samples or trolox standard were added and the absorbance was read at 754 nm. Results were expressed as mg Trolox equivalents per g of sample (mg TE/g) (Leite et al., 2011).

### 2.7. HPLC

Phenolic content analyses of non-digested and digested carob were performed by a reverse-phase (RP)-HPLC(Gunenc, HadiNezhad, Farah, Hashem, & Hosseinian, 2015). Samples were re-dissolved in methanol and filtered through 0.45 μm membrane PTFE filter, separated on an Alliance Waters 2695HPLC system (Waters Corp., Fisher Scientific, Milford, USA) equipped with a photodiode array detector (PDA, Waters 2998), Empower 3 software and auto sampler (Waters Corp., Milford, MA). Separations were carried out by means of an Atlantis R T3 column (150 mm × 4.6 mm, 5 μm particle size; Waters, Fisher Scientific, Milford, USA) using two solvents system: (A) 5 mL/L formic acid in milliQ water and (B) 100% acetonitrile. Phenolic compounds were analyzed at 30 °C, injecting 10 μL of sample, using a gradient elution at 1 mL/min according to the following gradient program: 0 min, 95% A; 0–35 min, 50% A; 35–40 min, 90% A and then return to 95% A in 10 min. The chromatograms were recorded at 280 for phenolic acids and 320 nm for flavonoids. The identification of the phenolic compounds was obtained by comparing the retention times with available external standards injected in the same conditions. Their quantification was carried out through calibration curves of the standards.

### 2.8. α-amylase inhibition

The α-amylase inhibition assay of both non-digested and digested carob was adapted from Telagari and Hullatti (2015) with some modifications. In brief, 50 μL of each sample at different concentrations (0.3, 0.5, 0.8 and 1 mg/mL) or negative control (distilled water) were pre-incubated with 10 μL of α-amylase (0.03 μkat/mL in 100 mmol/L sodium phosphate buffer pH 6.9) at 37 °C for 20 min. Then, 20 μL of 1% soluble starch solution (dissolved in 100 mmol/L sodium phosphate buffer pH 6.9) was added to the mixture as a substrate and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 100 μL of DNS (3, 5-dinitrosalicylicacid) reagent and boiled for 10 min. Absorbance (Abs) was read at 540 nm in an Epoch microplate reader (Biotek, Epoch, Fisher Scientific, Winooski, USA). Percent inhibition was calculated relative to the negative control having 100% enzyme activity (Telagari & Hullatti, 2015) as follows:

$$\text{Inhibitory activity (\%)} = [(Abs_{sample} - Abs_{control})/Abs_{control}] * 100$$

### 2.9. α-glucosidase inhibition

The α-glucosidase inhibition assays of both non-digested and digested carob were measured according to the method of Yao, Sang, Zhou, and Ren (2010). In a 96-well microplate, 50 μL of each sample at different concentrations (0.4, 0.5, 0.8 and 1 mg/mL) or negative control (distilled water) were reacted with 100 μL of α-glucosidase (0.03 μkat/mL dissolved in 100 mmol/L phosphate buffer, pH = 6.9) and pre-incubated at 37 °C for 20 min. Then, 50 μL of p-nitrophenyl-α-D-glucopyranoside (5 mmol/L in 100 mmol/L phosphate buffer, pH 6.9) was added to each well as a substrate and incubated at 37 °C for 5 min. After incubation, absorbance (Abs) was recorded at 405 nm by a microplate reader (Yao et al., 2010). The α-glucosidase inhibitory activity was calculated as:

$$\text{\% inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] * 100$$

### 2.10. In vitro fecal fermentation

The samples obtained after the intestinal digestion was centrifuged and the residue fraction (RF) was analyzed for colonic fermentation. Also, this part of the study was carried out in the Laboratoire de Microbiologie Appliquée (Bejaia, Algeria).

#### 2.10.1. Culture medium preparation

The culture medium was prepared as described by Zhou et al. (2016). The composition for 1 L of nutrient medium was 2.0 g peptone, 2.0 g yeast extract, 0.5 g L-cysteine, 0.5 g bile salts, 0.1 g NaCl, 0.04 g $KH_2PO_4$, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 6H_2O$, 2 g $NaHCO_3$, 1.0 mL resazurin solution (1%, w/v), 2.0 mL Tween-80, and 10 μL vitamin K. The growth medium was sterilized by autoclaving at 121 °C for 20 min (Zhou et al., 2016).

#### 2.10.2. Fecal slurry and fermentation

Fresh fecal samples were obtained from three healthy donors who reported no intestinal diseases and not received any antibiotic treatment during the last three months before the donation day. Samples were conserved at 4 °C and used within the 2 h of defecation. Fecal slurry was prepared by diluting feces in pre-sterilized phosphate buffered saline (PBS) solution (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L $Na_2HPO_4$ and 0.2 g/L $KH_2PO_4$, pH 7.3) to obtain 100 g/L fecal slurry to be used as the fermentation starter.

Then, 1 mL of the fecal slurry was added into 9 mL of culture medium containing 100 mg of RF obtained after the intestinal digestion step. Samples were incubated at 37 °C in an anaerobic incubator. Aliquots were taken out after 15 min, 5 h and 24 h fermentation, and submerged in ice bath to halt microbial activity before HPLC analysis as described above.

### 2.11. Bioaccessibility index

The bioaccessibility index represents the amount of phenolic compounds released after simulated gastrointestinal digestion or fecal fermentation that could become available for absorption into the systemic circulation. This index was determined as follows:

$$\text{Bioaccessibility index (\%)} = (A / B) \times 100$$

Where: A is the total phenol content (μg) in samples after *in vitro* digestion or fecal fermentation and B is the total phenol content (μg) in samples before *in vitro* digestion or fecal fermentation.

### 2.12. Statistical analysis

Statistical analysis was performed using IBM's SPSS Statistic version 24 software. Results were shown as the mean ± standard deviation

**Table 1**
TPC and TFC of undigested and digested carob fractions: *soluble free, soluble conjugated and insoluble-bound*[a].

| Analysis | Carob samples | Soluble Free | Soluble Conjugated | Bound | Total |
|---|---|---|---|---|---|
| **TPC** | Undigested | 15.4 ± 0.3[a] | 18.8 ± 0.1[a] | 29.1 ± 0.5[a] | 63.4 ± 0.3[a] |
| | Oral | 12.6 ± 0.4[a] | 16.0 ± 0.3[a] | 27.0 ± 0.4[a] | 55.7 ± 0.4[a] |
| | Gastric | 6.9 ± 0.4[b] | 7.2 ± 0.3[b] | 17.3 ± 0.2[b] | 31.5 ± 0.2[b] |
| | Intestinal | 11.1 ± 0.2[c] | 6.3 ± 0.4[b] | 9.3 ± 0.1[c] | 26.8 ± 0.2[c] |
| | **TPC (O+G+I)** | 30.6 ± 0.3 | 29.5 ± 0.3 | 53.6 ± 0.2 | 114.0 ± 0.3 |
| **TFC** | Undigested | 1.3 ± 0.7[a] | 1.4 ± 0.3[a] | 1.5 ± 0.3[a] | 4.3 ± 0.2[a] |
| | Oral | 0.8 ± 0.5[a] | 0.9 ± 0.1[a] | 1.4 ± 0.4[a] | 3.2 ± 0.3[a] |
| | Gastric | 0.6 ± 0.3[b] | 0.6 ± 0.1[b] | 0.8 ± 0.1[b] | 2.1 ± 0.1[b] |
| | Intestinal | 0.8 ± 0.5[c] | 0.5 ± 0.3[b] | 0.4 ± 0.2[c] | 1.8 ± 0.2[c] |
| | **TFC (O+G+I)** | 2.2 ± 0.3 | 2.0 ± 0.2 | 2.6 ± 0.2 | 7.1 ± 0.2 |

[a] Values are means of triplicates ± standard deviations and different letters in the same column indicate significant difference ($p < 0.05$) from Tukey's test. TPC (total phenolic content) values are milligram gallic acid equivalent per gram of sample (mg GAE/g sample): TFC (total flavonoid content) values are milligrams of rutin equivalent per gram of sample (mg RUE/g sample). O: oral phase, G: gastric phase, I: intestinal phase.

(SD) of triplicate experiments. The Kolmogorov-Smirnov test was applied to assess the normal distribution of the data. One-way analysis of variance (ANOVA) was applied for each parameter followed up with Tukey's post-hoc test for detecting significantly different means ($p < 0.05$).

## 3. Results and discussion

### 3.1. TPC and TFC of undigested carob

Total phenolic content (TPC) and total flavonoid content (TFC) of polyphenolic compounds (soluble free, soluble conjugated and bond) of undigested and *in vitro* digested carob samples were presented in Table 1. Both TPC and TFC values of undigested carob samples showed the predominance of bound phenolics. Indeed, the contribution of bound phenolics to TPC was 46% while soluble free and soluble conjugated phenolics contributed with 24% and 29%, respectively. Similar observations were also found in TFC with 36% contribution of bound fraction while soluble free and soluble conjugated flavonoids contributed with 31% and 32%, respectively. Those results were expected since the literature reported that the most dominant phenolic compounds are abundantly present in plant in their bound form then their free form (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2014). For instance, Wang et al. (2016) studied 14 selected bean samples and found the most phenolics in the bound fractions rather than free and conjugated forms (Wang et al., 2016). Similar results were also provided in the study of Gao, Ma, Wang, and Feng (2017) on seven commonly consumed vegetables in China (Gao et al., 2017). On the contrary, Sumczynski et al. showed a higher amount of TPC and TFC in the free fraction than the bound phenolics in commercial black and red rice (Sumczynski, Kotásková, Družbíková, & Mlček, 2016).

### 3.2. TPC and TFC of in vitro digested carob

The *in vitro* gastrointestinal digestion is a widely used method to determine the bioavailability of ingested compounds. In the present study, the carob pulp was submitted to an *in vitro* simulation model of human digestion and fecal fermentation to determine the bioaccessibility and the bioactivity of its phenolic compounds in soluble free, soluble conjugated and insoluble bound forms. Changes in TPC and TFC contents of soluble free, soluble conjugated and bound carob phenolics at different digestion stages were also presented in Table 1. As can be observed, the *in vitro* digestion affected differently the content of phenolics and flavonoids in the three fractions.

A minimal change during oral phase ($p > 0.05$) was noticed comparing to the initial content (undigested carob), more specifically, only 12% of losses for TPC and 25% for TFC were recorded. These results were expected and could be explained by the low contact time of the mouth phase (2 min) and the marginal effects of α-amylase (1.25 μkat/mL) as mentioned by (Mosele, Macià, Romero, & Motilva, 2016).

The gastric phase was deeply affecting ($p < 0.05$) both TPC and TFC of three fractional phenolics forms compared to undigested carob sample. The TPC values were decreased drastically by 45%, 38% and 56% in soluble free, soluble conjugated and bound fractions, correspondingly. Similarly, the TFC values were also decreased by 50% in free, 54% in conjugated and 49% in bound fractions.

At the end of intestinal phase, a marked increase ($p < 0.05$) was observed in soluble free phenolic content (11.1 mg GAE/g) and flavonoid content (0.8 RUE/g). This increase in both TPC and TFC suggests the main release of polyphenolic compounds from the carob powder in the intestinal digestion.

The increases in the amount of free phenolic compounds after digestion were verified by many researches such in vegetables juices (Wootton-Beard, Moran, & Ryan, 2011), cooked clove and nutmeg (Baker, Chohan, & Opara, 2013) and in persimmon fruit (Martínez-Las Heras, Pinazo, Heredia, & Andrés, 2017). This phenomenon could be the result of intestinal digestive enzymes and bile salts acting on the food matrix, facilitating the release of bound phenolics to the digestive juice (Zhang et al., 2017). On the other hand, these results are in contrast with the study of Ortega et al. (2011) and Ydjedd et al. (2017) who reported important decrease in free phenolic compounds after gastrointestinal digestion of carob flour (Ortega et al., 2011; Ydjedd et al., 2017). This is probably due to major differences in the matrix, its preparation and the used protocols.

By contrast, soluble conjugated phenolic and flavonoids content showed a significant decrease ($p < 0.05$) under gastrointestinal conditions to reach values of 6.3 mg GAE/g and 0.5 RUE/g respectively. Same observation was recorded for the TPC and TFC in bound fraction which decreased respectively ($p < 0.05$) to 9.3 mg GAE/g and 0.4 RUE/g compared with that before digestion (29.1 mg GAE/g and 1.5 mg RUE/g).

From these results, it can be concluded that the phenolic and flavonoid content insoluble conjugated and bound fractions tends to follow a different behavior than the free form along the digestion process of carob. The soluble conjugated and bound phenolic fraction demonstrated also different degree of decrease during digestion. These changes could be attributed to the release of conjugated and bound compounds from the carob under the effect of digestive enzymes.

**Table 2**
Phenolic profiles of undigested carob in soluble free, soluble conjugated and bound fractions[a].

| | Phenolic compounds | Soluble Free | Soluble Conjugated | Bound |
|---|---|---|---|---|
| Phenolic acids | Gallic acid | 162.5 ± 0.1a | 639.3 ± 0.1b | 1233.0 ± 0.1c |
| | Proto-catechuic acid | 39.2 ± 0.4$^{a}$ | 75.1 ± 0.1$^{b}$ | 226.0 ± 0.6$^{c}$ |
| | Chlorogenic acid | 54.1 ± 0.1$^{a}$ | 66.2 ± 0.6$^{a}$ | 523.3 ± 0.2$^{b}$ |
| | Vanillic acid | Nd | 7.4 ± 0.4$^{a}$ | 15.4 ± 0.4$^{a}$ |
| | Caffeic acid | Nd | 5.2 ± 0.1$^{a}$ | 14.3 ± 0.6$^{a}$ |
| | Syringic acid | Nd | 6.2 ± 0.9$^{a}$ | 16.0 ± 0.1$^{a}$ |
| | p-coumaric acid | 12.7 ± 0.1$^{a}$ | 24.7 ± 0.1$^{b}$ | 5.3 ± 0.2$^{c}$ |
| | Ferulic acid | 13.4 ± 0.1$^{a}$ | 117.5 ± 0.1$^{b}$ | 8.4 ± 0.1$^{a}$ |
| | o-coumaric acid | 11.0 ± 0.4$^{a}$ | 48.0 ± 0.1$^{b}$ | 142.4 ± 0.1$^{c}$ |
| | *Trans-* cinnamic acid | 42.4 ± 0.1$^{a}$ | 53.0 ± 0.6$^{a}$ | 82.0 ± 0.1$^{b}$ |
| | **Total phenolic acids (TPA)** | **335.3 ± 0.9$^{a}$** | **1042.6 ± 0.4$^{b}$** | **2266.1 ± 0.8$^{c}$** |
| Flavonoids | (+)-catechin | 11.0 ± 0.3$^{a}$ | 24.4 ± 0.5$^{b}$ | 138.4 ± 0.2$^{c}$ |
| | Rutin | 118.3 ± 0.2$^{a}$ | 155.8 ± 0.1$^{b}$ | 89.1 ± 0.1$^{c}$ |
| | Myricetin | 18.1 ± 0.4$^{a}$ | 19.0 ± 0.1$^{a}$ | 8.2 ± 0.2$^{b}$ |
| | Isoquercitrin | 24.5 ± 0.6$^{a}$ | 42.8 ± 0.2$^{b}$ | 104.5 ± 0.4$^{c}$ |
| | Apigenin | 3.3 ± 0.8$^{a}$ | Nd | 10.6 ± 0.6$^{b}$ |
| | Kaempferol | Nd | Nd | 16.7 ± 0.1$^{a}$ |
| | **Total flavonoids (TF)** | **175.2 ± 0.1$^{a}$** | **242.0 ± 0.1$^{b}$** | **367.5 ± 0.1$^{c}$** |
| | **Total phenolics (TPA + TF)** | **510.5 ± 0.2$^{a}$** | **1284.6 ± 0.1$^{b}$** | **2633.6 ± 0.2$^{c}$** |

[a] Values are means of triplicates ± standard deviations (µg/g of sample) and different letters in the same row indicate significant difference ($p < 0.05$) from Tukey's test. Nd: not detected.

### 3.3. Phenolic profiles of undigested carob

The phenolic profile and their contents in soluble free, soluble conjugated and bound fractions of undigested carob were investigated by a RP-HPLC-DAD based on matching their retention time and UV absorbance spectra (280 and 320 nm) with respective standards. The results were represented in Table 2. Ten major phenolic acids corresponding to gallic, proto-catechuic, chlorogenic, vanillic, syringic, *p*-coumaric, caffeic, *o*-coumaric, ferulic and *trans*-cinnamic acids were detected in soluble (free and conjugated) and insoluble (bound) fractions of undigested carob. Similarly, six flavonoids were also found in these fractions and assigned to (+)-catechin, rutin, myricetin, isoquercitrin, apigenin and kaempferol.

As observed earlier in TPC analysis (section 3.1), the bound form revealed higher ($p < 0.05$) amount of phenolics than the other two fractions with a total of 2633 µg/g of sample. Gallic acid was the most abundant phenolic acid in all carob fractions and its amount increased significantly ($p < 0.05$) after alkaline hydrolysis from 162.5 µg/g in free form to 1233 µg/g in bound form (Table 2). This finding was parallel to several authors (Benković, Belščak-Cvitanović, Bauman, Komes, & Srečec, 2017; Roseiro et al., 2013) who reported the predominance of gallic acid in carob flour. No vanillic, caffeic or syringic acid was detected in the free fraction, whereas they were released in the conjugated and bound fractions. With respect to flavonoids, rutin was mainly present in the free fraction whereas (+)-catechin and isoquercitrin were most detected in the soluble conjugated and bound fractions.

There is limited number of studies in the compositional analysis of phenolics for carob plant (in free, conjugated and bound forms). To our knowledge, only Torun, Ayaz, Colak, Grúz, and Strnad (2013) conducted a study on the composition of free, ester and glycosides phenolics of natural and commercial carob flour. This same study reported the presence of six phenolic acids concentrated for most of them in the free fraction (Torun et al., 2013).

### 3.4. Phenolic profiles of digested carob and their bioaccessibility index

The effects of *in vitro* gastrointestinal digestion on bioaccessibility of carob phenolic compounds in the three fractions were evaluated in the present study and results are summarized in Table 3. Samples from each *in vitro* digestion steps were evaluated for their polyphenolic contents in soluble free, soluble conjugated and bound forms. In addition, depending on the available standards (mentioned in section 3.3), the main focus of this study was to measure only the free or hydrolysed forms of phenolics released after the *in vitro* digestion or the fecal fermentation.

Total phenolic acid (TPA) and total flavonoid (TF) contents of carob fractions after digestion showed similar trends as TPC and TFC results (Table 1). The polyphenolic content in the carob free fraction demonstrated a significant increase ($p < 0.05$) after the gastrointestinal digestion with reference to the initial carob values (Table 2). With regard to the individual phenolic, gallic and chlorogenic acids remained the most bioaccessible phenolic acids with percentages of 647.4% and 485.4% respectively. However, ferulic acid demonstrated a gradual reduce ($p < 0.05$) under the adopted conditions with only 31.2% of bioaccessibility. The increment in gallic acid concentration could be due to the hydrolysis of galloylated molecules as hydrolysable tannins (gallotannins) present in the carob matrix under *in vitro* digestion conditions. This result is supported by the study of Celep, İnan, Akyüz, and Yesilada (2017) who reported increment in the concentration of gallic and cholorogenic acid in *Hypericum perfoliatum* plant after intestinal digestion (Celep et al., 2017). Also another study (Farah, Monteiro, Donangelo, & Lafay, 2008) reported the high bioaccessibilty of cholorogenic acid in green coffee and its rapid metabolism in humans.

The free flavonoids, (+)-catechin and rutin showed the highest bioaccessibility with 558.3% and 267.2%, respectively. This increase in the amount of flavonoids values is may be related to the hydrolysis of the complex compounds (as galloylated catechins) from their glycoside to aglycone forms (Ortega et al., 2011). However, isoquercitrin was degraded gradually (39.1%). Same results were found by (Luzardo-Ocampo et al., 2017) who reported a reduction in isoquercitrin concentration at the end of the intestinal phase in digestion of both corn and common bean chips. Also, another study (Ortega et al., 2011) reported the complete degradation of isoquercitrin in carob flour. In another hand, the reason for some compounds having more than 100% bioaccessibility value is due to their increased amounts under the *in-vitro* digestion conditions compared to their free amount in the initial condition (before digestion). Indeed, the complex polyphenols both conjugated and bound (for example the gallotannins) of carob pulp will be released under the digestive enzymes activity. As a result, the amount of free phenolic will increase (i.e, gallic acid) and exceed the initial free amount quantified in the carob powder.

**Table 3**
Phenolic profiles of carob and their bioaccessibility during the *in vitro* gastrointestinal digestion[a].

| | | Phenolic compound | Oral | Gastric | Intestinal | Bioaccessibility (%) |
|---|---|---|---|---|---|---|
| **Soluble free** | Phenolic acids | Gallic acid | 152 ± 8$^{a}$ | 88 ± 2$^{b}$ | 984 ± 16$^{c}$ | 647.4 |
| | | Proto-catechuic acid | 32.1 ± 0.4$^{a}$ | 5.6 ± 0.4$^{b}$ | 113 ± 14$^{c}$ | 352.0 |
| | | Chlorogenic acid | 48 ± 0.8$^{a}$ | 15.2 ± 0.3$^{b}$ | 233 ± 20$^{c}$ | 485.4 |
| | | Vanillic acid | Nd | 4.2 ± 0.9$^{a}$ | 42 ± 8$^{b}$ | Nd |
| | | Caffeic acid | Nd | 1.7 ± 0.5$^{a}$ | 15 ± 6$^{b}$ | Nd |
| | | Syringic acid | Nd | 1.1 ± 0.6$^{a}$ | 53 ± 7$^{b}$ | Nd |
| | | p-coumaric acid | 11.9 ± 0.6$^{a}$ | 4.5 ± 0.1$^{b}$ | 46 ± 15$^{c}$ | 386.5 |
| | | Ferulic acid | 14.7 ± 0.7$^{a}$ | 10 ± 1$^{a}$ | 4.6 ± 0.8$^{b}$ | 31.2 |
| | | o-coumaric acid | 11.0 ± 0.6$^{a}$ | 10.2 ± 0.7$^{a}$ | 27 ± 7$^{b}$ | 245.4 |
| | | *Trans-* cinnamic acid | 39.4 ± 0.6$^{a}$ | 15.8 ± 0.6$^{b}$ | 49 ± 11$^{c}$ | 124.4 |
| | | **Total phenolic acids (TPA)** | **309 ± 1$^{a}$** | **156 ± 4$^{b}$** | **1566 ± 19$^{c}$** | Nd |
| | Flavonoids | (+)-catechin | 12.0 ± 0.4$^{a}$ | 6.1 ± 0.3$^{b}$ | 67 ± 21$^{c}$ | 558.3 |
| | | Rutin | 110 ± 10$^{a}$ | 82 ± 11$^{b}$ | 294 ± 17$^{c}$ | 267.2 |
| | | Myricetin | 16 ± 1$^{a}$ | 5.5 ± 0.1$^{b}$ | 32 ± 9$^{c}$ | 200.0 |
| | | Isoquercitrin | 23.0 ± 0.9$^{a}$ | 16 ± 2$^{b}$ | 9 ± 12$^{c}$ | 39.1 |
| | | Apigenin | 3.3 ± 0.4$^{a}$ | 3.0 ± 0.1$^{a}$ | 8 ± 6$^{b}$ | 242.4 |
| | | Kaempferol | Nd | Nd | 12 ± 7 | Nd |
| | | **Total flavonoids (TF)** | **164 ± 9$^{a}$** | **112 ± 7$^{b}$** | **422 ± 12$^{c}$** | **Nd** |
| | | **Total phenolics (TPA+TF)** | **473 ± 10$^{a}$** | **268 ± 9$^{b}$** | **1988 ± 12$^{c}$** | **Nd** |
| **Soluble Conjugated** | Phenolic acids | Gallic acid | 616 ± 24$^{a}$ | 425 ± 21$^{b}$ | 331 ± 18$^{c}$ | 53.7 |
| | | Proto-catechuic acid | 64 ± 11$^{a}$ | 41 ± 8$^{b}$ | 18 ± 1$^{c}$ | 28.1 |
| | | Chlorogenic acid | 57 ± 9$^{a}$ | 27 ± 5$^{b}$ | 19 ± 2$^{c}$ | 33.3 |
| | | Vanillic acid | 6.0 ± 0.1$^{a}$ | 5 ± 1$^{a}$ | Nd | Nd |
| | | Caffeic acid | 4.4 ± 0.1$^{a}$ | 4 ± 1$^{a}$ | Nd | Nd |
| | | Syringic acid | 6.0 ± 0.1$^{a}$ | 5.0 ± 0.1$^{a}$ | 4 ± 1$^{a}$ | 66.6 |
| | | p-coumaric acid | 19.2 ± 2.4$^{a}$ | 9 ± 0.1$^{b}$ | 4.0 ± 0.4$^{c}$ | 21.0 |
| | | Ferulic acid | 109 ± 7$^{a}$ | 65 ± 7$^{b}$ | 28 ± 4$^{c}$ | 25.7 |
| | | o-coumaric acid | 43 ± 9$^{a}$ | 13 ± 6$^{b}$ | 4 ± 0.1$^{c}$ | 9.30 |
| | | *Trans-* cinnamic acid | 49 ± 7$^{a}$ | 34 ± 10$^{b}$ | 21 ± 0.1$^{c}$ | 42.8 |
| | | **Total phenolic acids (TPA)** | **974 ± 10$^{a}$** | **628 ± 7$^{b}$** | **429 ± 8$^{c}$** | |
| | Flavonoids | (+)-catechin | 18 ± 3$^{a}$ | 15 ± 7$^{a}$ | Nd | Nd |
| | | Rutin | 138 ± 10$^{a}$ | 102 ± 18$^{b}$ | 75 ± 12$^{c}$ | 54.3 |
| | | Myricetin | 17 ± 11$^{a}$ | 12 ± 6$^{b}$ | 4 ± 0.5$^{c}$ | 23.5 |
| | | Isoquercitrin | 40 ± 4$^{a}$ | 28 ± 7$^{b}$ | 17 ± 1$^{c}$ | 42.5 |
| | | Apigenin | Nd | 7.2 ± 0.1 | Nd | Nd |
| | | Kaempferol | Nd | Nd | Nd | Nd |
| | | **Total flavonoids (TF)** | **213 ± 11$^{a}$** | **164 ± 10$^{b}$** | **96 ± 9$^{c}$** | |
| | | **Total phenolics (TPA+TF)** | **1187 ± 12$^{a}$** | **792 ± 9$^{b}$** | **525 ± 7$^{c}$** | |
| **Insoluble bound** | Phenolic acids | Gallic acid | 1154 ± 25$^{a}$ | 854 ± 22$^{b}$ | 628 ± 21$^{c}$ | 54.5 |
| | | Proto-catechuic acid | 193 ± 18$^{a}$ | 98 ± 15$^{b}$ | 71 ± 14$^{c}$ | 36.8 |
| | | Chlorogenic acid | 487 ± 20$^{a}$ | 270 ± 14$^{b}$ | 185 ± 10$^{c}$ | 35.28 |
| | | Vanillic acid | 11 ± 6$^{a}$ | 6 ± 1$^{b}$ | Nd | Nd |
| | | Caffeic acid | 12 ± 5$^{a}$ | 5.0 ± 0.1$^{b}$ | Nd | Nd |
| | | Syringic acid | 15 ± 5$^{a}$ | 8.0 ± 0.1$^{b}$ | 7.0 ± 0.1$^{b}$ | 46.6 |
| | | p-coumaric acid | 3.2 ± 0.6 | Nd | Nd | Nd |
| | | Ferulic acid | 7 ± 2$^{a}$ | 4.0 ± 0.6$^{a}$ | 1.9 ± 0.5$^{b}$ | 27.1 |
| | | o-coumaric acid | 131 ± 10$^{a}$ | 108 ± 11$^{b}$ | 86 ± 11$^{c}$ | 65.6 |
| | | *Trans-* cinnamic acid | 70 ± 7$^{a}$ | 59 ± 12$^{b}$ | 33 ± 8$^{c}$ | 47.1 |
| | | **Total phenolic acids (TPA)** | **2083 ± 12$^{a}$** | **1412 ± 11$^{b}$** | **1012 ± 8$^{c}$** | |
| | | (+)-catechin | 114 ± 11$^{a}$ | 71 ± 8$^{b}$ | 57 ± 10$^{c}$ | 50.0 |
| | | Rutin | 72 ± 14$^{a}$ | 61 ± 10$^{a}$ | 48 ± 10$^{b}$ | 66.7 |
| | Flavonoids | Myricetin | 2.4 ± 0.1 | Nd | Nd | Nd |
| | | Isoquercitrin | 88 ± 12$^{a}$ | 51 ± 11$^{b}$ | 30 ± 1$^{c}$ | 34.1 |
| | | Apigenin | 6.0 ± 0.5 | Nd | Nd | Nd |
| | | Kaempferol | 11 ± 0.4 | Nd | Nd | Nd |
| | | **Total flavonoids (TF)** | **293 ± 5$^{a}$** | **183 ± 7$^{b}$** | **135 ± 7$^{c}$** | Nd |
| | | **Total phenolics (TPA+TF)** | **2376 ± 11$^{a}$** | **1595 ± 12$^{b}$** | **1147 ± 11$^{c}$** | **Nd** |

[a] Values are means of triplicates ± standard deviations (μg/g of sample). Means followed by the different letters (a,b,c) in the same line represent a statistically significant difference ($p < 0.05$). Nd: not detected/determined.

The conjugated and bound polyphenols were affected differently by the digestion conditions in each step. In fact, their total amounts (in mouth + gastric + intestinal phases) were decreased with a loss of 55.7% and 51.7%, respectively. Again, gallic acid remained the most abundant compound in both fractions while vanillic, caffeic, apigenin and kaempferol were not quantifiable after intestinal digestion. (+)-catechin and myricetin were also completely degraded in conjugated and bounds flavonoids respectively. These findings are in agreement with the work conducted by Juániz et al. (2016) who reported a reduce in bound phenolics from 34% to 11% after the

**Table 4**
Antioxidant capacity of soluble free, soluble conjugated and bound phenolics fractions of both undigested and digested carob[a].

| Analysis | Carob samples | Soluble Free | Soluble Conjugated | Bound |
|---|---|---|---|---|
| DPPH | Undigested | $75 \pm 7^a$ | $102 \pm 12^a$ | $190 \pm 10^a$ |
| | Oral | $70 \pm 10^a$ | $107 \pm 11^a$ | $161 \pm 8^b$ |
| | Gastric | $60 \pm 7^b$ | $91 \pm 8^a$ | $84 \pm 8^c$ |
| | Intestinal | $107 \pm 12^c$ | $59 \pm 8^b$ | $66 \pm 10^d$ |
| ABTS | Undigested | $223 \pm 10^a$ | $365 \pm 20^a$ | $414 \pm 12^a$ |
| | Oral | $194 \pm 9^a$ | $315 \pm 10^a$ | $386 \pm 12^a$ |
| | Gastric | $134 \pm 9^b$ | $289 \pm 14^a$ | $277 \pm 9^b$ |
| | Intestinal | $399 \pm 9^c$ | $79 \pm 11^b$ | $89 \pm 7^c$ |
| ORAC | Undigested | $315 \pm 12^a$ | $419 \pm 21^a$ | $494 \pm 14^a$ |
| | Oral | $277 \pm 11^a$ | $354 \pm 14^b$ | $389 \pm 12^b$ |
| | Gastric | $168 \pm 11^b$ | $203 \pm 8^c$ | $246 \pm 10^c$ |
| | Intestinal | $415 \pm 11^c$ | $70 \pm 7^d$ | $102 \pm 11^d$ |

[a] Values are means of triplicates ± standard deviation (SD). Means followed by the different letters within a column are significantly different ($p < 0.05$). DPPH are expressed as mg Gallic equivalents/g; ORAC are expressed as µmol Trolox equivalents/g; ABTS are expressed as mg Trolox equivalents/g.

gastrointestinal digestion in raw green pepper (Juániz et al., 2016). The same authors reported also the degradation of free and bounds polyphenols in raw and cooked cardoon when subjected to simulated digestion.

Also, it should be noted that, despite the decrease in concentration of bound phenolics during the gastrointestinal digestion, their degradation remains partial. In this regard, Adom and Liu (2002) reported that insoluble bound phenolics can resist the gastric and intestinal digestion to reach the large intestine since cell wall component are difficult to be digested (Adom & Liu, 2002). Also Kroon, Faulds, Ryden, Robertson, and Williamson (1997) indicated that only 2.6% of ferulic acid was released from wheat under gastric and intestinal conditions (Kroon et al., 1997).

### 3.5. Antioxidant capacity

In the present work, three different assays were carried out to measure the changes in carob antioxidant capacity induced during the digestion process. These methods were based on two different chemical mechanisms: the scavenging of the free radical DPPH and ABTS and oxygen radical absorbance capacity ORAC. Results illustrated in Table 4 represent the variation of antioxidant capacity in carob soluble free, esterified and insoluble-bound phenolic fractions before and after simulated gastrointestinal digestion stages.

DPPH, ABTS, and ORAC values of carob phenolics in soluble free forms presented a great increase ($p < 0.05$) to reach respectively 107 mg GAE/g, 399 mg TE/g and 415 µmol TE/g under gastrointestinal conditions with reference to undigested extract. A similar trend (increase of the antioxidant capacity during gastrointestinal digestion) has been reported in the literature, for instance, DPPH and ABTS of different fruits were observed to be significantly increased after digestion process (Chen, Zhao, Luo, Li, & Gao, 2014). Likewise, a study of Pineda-Vadillo et al. (2016) published a high ORAC value of intestinal digested strawberry and peach yoghurt (Pineda-Vadillo et al., 2016). However, other previous studies indicated a loss in antioxidant capacity after intestinal digestion (Burgos-Edwards, Jiménez-Aspee, Thomas-Valdés, Schmeda-Hirschmann, & Theoduloz, 2017; Gullon et al., 2015).

The higher antioxidant capacity observed after the digestive process could be attributed to pH changes and the deprotonation of the hydroxyl moieties present on the aromatic rings of the phenolic compounds (Bouayed, Hoffmann, & Bohn, 2011). It might be related also to the structural changes of phenolic molecules or liberation of new compounds having higher antioxidant capacity (Pineda-Vadillo et al., 2016).

Antioxidant capacity in esterified and bound fractions changed differently during the digestion process. No significant differences ($p > 0.05$) was found in DPPH and ABTS values released from conjugated form after oral and gastric stages whereas a significant decrease ($p < 0.05$) was observed after intestinal phase (59 mg GAE/g and 79 mg TE/g). The ORAC values in conjugated form decreased gradually during the gastrointestinal simulation reaching 70 µmol TE/g. The same trend of decrease was also seen following the gastrointestinal digestion in the antioxidant capacity of bound fraction.

The results obtained in this work confirmed that carob extract containing high phenolic and flavonoid amounts had strongest antioxidant capacity. Indeed, several studies from scientific literature reported a relationship between polyphenolic compounds and antioxidant capacity. Coefficients of correlation (r) were calculated to explain the relationship between TPC and TFC of carob fractions and their antioxidant values (DPPH, ABTS and ORAC).

In oral digestion, the three carob fractions showed positive and strong correlation ($r > 0.994$) between TPC and TFC and the antioxidant capacity measured with DPPH, ABTS and ORAC assays. The results obtained in gastric phase demonstrated a good correlation between TPC and the antioxidant capacity measured in free, conjugated and insoluble bound fractions. However, this correlation was moderate in case of TFC. At the end of intestinal digestion, again the DPPH, ABTS and ORAC values found in the three carob fractions were strongly consistent with TPC and TFC. These results are parallel to several previous studies (Gullon et al., 2015; Lucas-Gonzalez et al., 2016) which reported a high correlation between polyphenolic compounds and antioxidant capacity.

### 3.6. Fecal fermentation

Undigested polyphenols particularly those covalently bound to food matrix resist to digestive enzymes and reach the large intestine where they could be metabolized by colonic microbiota to become available for absorption. In the present work, the residual fraction (RF) of carob samples obtained after gastrointestinal digestion was submitted to fecal fermentation. The amount (µg/g of sample) and bioaccessibility percentage of polyphenols released during this process are listed in Table 5. Results showed an important microbial metabolic activity; the concentration of main phenolic compounds demonstrated a gradual increase during the first 5 h of fermentation, whilst their amounts were rapidly degraded after 24 h. Myricetin (79.5%) and gallic acid (20.0%) were the most abundant metabolites. The minor phenolics (e.g. vanillic and caffeic acids, kaempferol) were completely metabolized at the end of incubation period since they were not detected.

This is consistent with the recent finding of Mosele et al. (2016) who reported an accumulation of gallic acid at early stages of incubation (2 h) and then its total degradation (24 h) (Mosele et al., 2016). Similarly, Alqurashi et al. (2017) reported also the decrease or the complete degradation of phenolic acids in acai after 24 h of fermentation (Alqurashi et al., 2017). Juániz et al. (2016) found also a quick metabolization of isoquercitrin and its derivatives during the first minutes of the colonic biotransformation of raw green pepper (Juániz et al., 2016).

The metabolism of polyphenols, in particular the bounds one in the colon is due to enzymatic release (esterase and xylanase activities) of the existing microflora (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014). In parallel, some new peaks (not identified because of unavailable standards) were observed during the fecal fermentation probably due to the generation of new microbial metabolites.

### 3.7. The α-amylase and α-glucosidase inhibitory activities

To the best of our knowledge, no studies have compared the antidiabetic activities in carob during the gastrointestinal digestion. Figs. 1

**Table 5**
Phenolic compounds contents in carob (indigested fraction) during fecal fermentation[a].

| | Phenolic compounds | Control[1] | 15 min | 5 h | 24 h | Bioaccessibility (%) |
|---|---|---|---|---|---|---|
| **Phenolic acids** | Gallic acid | 641 ± 10[a] | 686 ± 11[b] | 711 ± 9[c] | 128 ± 4[d] | 20.0 |
| | Proto-catechuic acid | 143 ± 0.6[a] | 151.0 ± 0.1[a] | 86.0 ± 0.1[b] | 22.0 ± 0.2[c] | 15.4 |
| | Chlorogenic acid | 550 ± 12[a] | 570 ± 10[a] | 601 ± 10[b] | 68 ± 5[c] | 12.3 |
| | Vanillic acid | 31.0 ± 0.4 | Nd | Nd | Nd | Nd |
| | Caffeic acid | 10 ± 1[a] | 0.5 ± 0.1[b] | Nd | Nd | Nd |
| | Syringic acid | 20 ± 2[a] | 2.4 ± 0.1[b] | Nd | Nd | Nd |
| | p-coumaric acid | 34 ± 4[a] | 20.3 ± 0.1[b] | 8.7 ± 0.5[c] | Nd | Nd |
| | Ferulic acid | 14.4 ± 0.5[a] | 8.7 ± 0.8[b] | Nd | Nd | Nd |
| | o-coumaric acid | Nd | 6.7 ± 0.1[a] | 7.5 ± 0.1[a] | 10 ± 0.1[a] | Nd |
| | *Trans*- cinnamic acid | 11.7 ± 0.2 | Nd | Nd | Nd | Nd |
| | **Total phenolic acids (TPA)** | **1455 ± 12[a]** | **1446 ± 10[a]** | **1414 ± 9[b]** | **228 ± 9[c]** | |
| **Flavonoids** | (+)-catechin | 60 ± 0.4[a] | 71 ± 0.1[a] | 30.1 ± 0.1[b] | Nd | Nd |
| | Rutin | 104 ± 0.2[a] | 62.0 ± 0.1[b] | 41 ± 0.1[c] | 20.0 ± 0.1[d] | 19.2 |
| | Myricetin | 20.5 ± 0.1[a] | 35 ± 0.1[a] | 41.5 ± 0.1[a] | 16.3 ± 0.1[b] | 79.5 |
| | Isoquercitrin | 42 ± 6[a] | 26 ± 1[b] | Nd | Nd | Nd |
| | Apigenin | Nd | Nd | Nd | Nd | Nd |
| | Kaempferol | 0.05 ± 0.14 | Nd | Nd | Nd | Nd |
| | **Total Flavonoid (TF)** | **226 ± 10[a]** | **194 ± 9[b]** | **112 ± 8[c]** | **36 ± 4[d]** | |
| | **Total phenolics (TPA+TF)** | **1681 ± 11[a]** | **1640 ± 8[b]** | **1526 ± 9.47[c]** | **264 ± 6[d]** | |

[a ,1]: indigested fraction obtained after the gastrointestinal digestion of carob pulp. Different letters for each row indicate significant differences ($p < 0.05$). Nd: not detected/determined. Values are means of triplicates ± standard deviations (μg/g of sample).

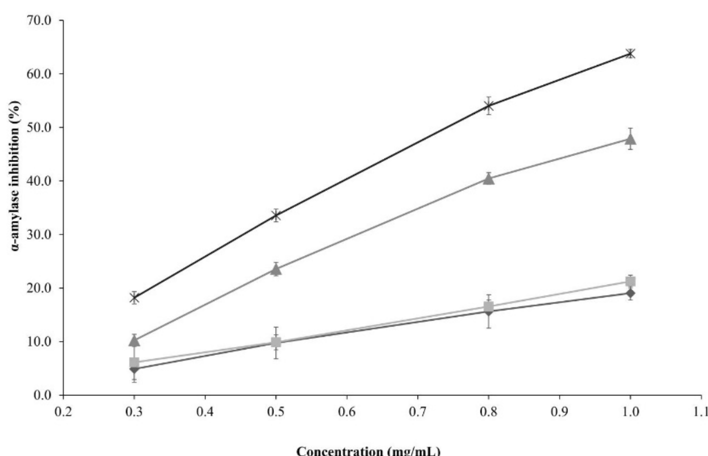


**Fig. 1.** Effects of different concentrations (0.3, 0.5, 0.8, and 1 mg/mL) of undigested carob and its in-vitro digested fractions on α-amylase inhibitory activities. (Legend:◆Undigested carob, oral fraction,■ Gastric fraction,▲Intestinal fraction).

and 2 displayed the inhibitory effect of carob powder and its digested fractions on α-amylase and α-glucosidase activities.

It is clear that the gastric and intestinal fractions were more active and increased significantly the α-amylase inhibitory activity of carob. Both undigested carob and its digested fractions showed a positive dose-dependent inhibition of α-amylase activity (0.3–1.0 mg/mL). No significant differences ($p > 0.05$) in α-amylase inhibitory activity were observed between oral digestion and undigested carob. The intestinal fraction demonstrated the highest α-amylase inhibition with a rate of 64% at the concentration of 1 mg/mL. In this way, Silva, Sampaio, Freitas, & Torres, 2017 studied the inhibitory effect of polyphenols from guaraná and reported a rate > 60% after digestion (Silva, Sampaio, Freitas, & Torres, 2017). This might be due to the mechanism of inhibition by the capacity of polyphenols to bind and precipitate digestive enzymes as explained in a previous study (He, Lv, & Yao, 2007).

The α-glucosidase inhibitory of phenolic compounds was also increased significantly after gastric and intestinal digestion, but this activity was not linear to phenolics concentration. In other words, the percentage of inhibition decreases when the concentration of phenolic extract is high. Similar trend was observed in the study of (Silva, Sampaio, Freitas, & Torres, 2017) who reported that the effect of guaraná extract on α-glucosidase activity was not positively related to its concentration. Again, the intestinal fraction exhibited stronger inhibitory effect with a percentage of 42% at the concentration of 0.4 mg/mL. This results were lower than those reported by (Silva, Sampaio, Freitas, & Torres, 2017) who found 90% inhibition of guaraná extract for the same concentration.

As mentioned above, the *in vitro* digestion can affect greatly the content and ingredients of the food matrix. Thus, it can induce great contribution to inhibitory activity of α-amylase and α-glucosidase. The high inhibitory activities of intestinal fractions found in our study coincide with the high polyphenolics content in these fractions (Table 1). This let to suggest that phenolic compounds identified in the carob after digestion might influence glucose metabolism by inhibiting

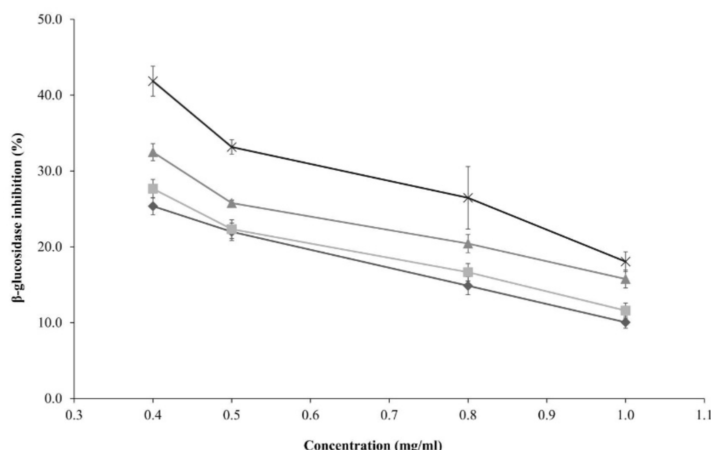


**Fig. 2.** Effects of different concentrations (0.4, 0.5, 0.8, and 1 mg/mL) of undigested carob and its in-vitro digested fractions on α-glucosidase inhibitory activities. (Legend: ◆Undigested carob,■oral fraction,▲Gastric fraction,XIntestinal fraction).

carbohydrate digestion.

## 4. Conclusion

In summary, the phenolic compounds in free, conjugated and bound fractions of carob pulp were significantly affected by the *in vitro* gastrointestinal digestion. Indeed, free phenolics were highly increased while the conjugated and bound phenolics were partially degraded over time under digestion conditions. Meanwhile, these changes resulted in liberation of compounds with high antioxidant capacity and increasing ability to inhibit enzymes involved in carbohydrate metabolism. Moreover, many of carob compounds remained non digestible reaching colon where they are metabolized to exert their health benefits. The results from this work clearly extend the knowledge in conjugated and bound phenolic composition and the effect of digestion process on health promoting properties of carob. However, some restrictions were noted such as very limited information in literature about carob phenolic profiles (soluble and non-soluble fractions during digestion or non-digested carob), as well as the limit of the available standards. Further studies (mass spectrometric detections) are required to fully understand the chemical structure modifications and identifications of bioactive compounds released after the digestion and the metabolites resulted under the microbial fermentation; specifically that the digested fractions had demonstrated highest inhibitory activity on α-amylase and α-glucosidase.

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**Abstract**

In the present study, novel synbiotic fermented milk was developed using local starter and probiotic bacteria with carob powder as a prebiotic ingredient. A total of 110 bacterial strains belonging to *Lactococcus* and *Lactobacillus* genera were isolated from Algerian artisanal cheeses and investigated for their starter and probiotic potentialities. All strains were first screened for their antibacterial activity against two foodborne pathogens, *Escherichia coli* and *Staphylococcus aureus* using the spot and agar well diffusion tests. Based on this screening, 5 lactococci and 14 lactobacilli isolates were selected and furthermore, studied for their technological and probiotic traits. One (1) lactococci isolate (C15) showed interesting technological potential such high milk acidification, good lipolytic and proteolytic activities. Two (2) lactobacilli isolates (B13 and B38) exhibited encouraging survival rates under gastrointestinal tract conditions (8.20, 7.27 CFU/mL) as well as strong ability to adhere to HT-29 cells (84 and 74 %), high antioxidant capacity and good technological activities. The selected isolates were identified by 16S rDNA sequencing as *Lactococcus (Lc.) lactis* and *Lactobacillus (Lb.) brevis*. In another hand, the study of the carob's composition in polyphenols showed high content of phenolic acids and flavonoids present in free, conjugated and bound form with gallic acid and rutin being the most abundant ones. When the carob powder was submitted to a simulated gastrointestinal digestion, the polyphenols were released and became more accessible leading to an increase in the antioxidant capacity. By combining the starter *Lc. lactis* C15, both probiotics *Lb. brevis* B13 and B38 with carob powder at 4%, a successfully synbiotic fermented milk was developed. Physicochemical characteristics, probiotic viability, total phenolic content (TPC) and antioxidant capacity as well as the hypoglycemia activity of the carob fermented milk were determined during cold storage (28 days/4°C) and gastro-intestinal digestion. Carob powder addition to fermented milk improved the growth of *Lb. brevis* strains and maintained their viability during the storage period (8 log CFU/g) and after digestion (7 log CFU/g). Carob fermented milk displayed high TPC and high antioxidant capacity during the storage. The *in vitro* digestion resulted in the release of bioaccessible phenolics where gallic acid (441%) and (+)-catechin (486%) were the most quantified phenolic compounds; thus, the inhibition of α-amylase (52%) and α-glucosidase (37%) activities. These results demonstrate the potential of carob fermented milk as a functional food to be an important source of viable probiotics and bioaccessible polyphenols giving health benefits.

**Key words:** Fermented milk, synbiotic, *Lactococcus lactis*, *Lactobacillus brevis*, carob, gastrointestinal digestion, polyphenols, antioxidant activity, hypoglycemia activity.



**Résumé**

L'objectif de la présente étude était de développer un nouveau lait fermente synbiotique en utilisant un levain et des probiotiques local combiné avec la poudre de la caroube comme ingrédient prébiotique. Un total de 110 souches bactériennes appartenant aux genres *Lactococcus* et *Lactobacillus* ont été isolées de fromages artisanaux algériens et étudiées pour leur caractéristiques levain et probiotiques. Tout d'abord, les souches ont été criblées à base de leur activité antibactérienne à l'égard de deux agents pathogènes, *Escherichia coli* et *Staphylococcus aureus* en utilisant le test des spots et le test des puits. A l'issue de ce pré-criblage, 5 (cinq) isolats de lactocoques et 14 lactobacilles ont été sélectionnés et, en outre, étudiés pour leurs caractéristiques technologiques et probiotiques. Un (1) isolat de lactocoques (C15) a montré un potentiel technologique intéressant comme une acidification élevée du lait, de bonnes activités lipolytiques et protéolytiques. Deux (2) isolats de lactobacilles (B13 et B38) présentaient des taux de survie important sous les conditions du tractus gastro-intestinal (8.20, 7.27 UFC/mL) ainsi qu'une forte capacité à adhérer aux cellules HT-29 (84 et 74%), une capacité antioxydante élevée et de bonnes activités technologiques. Ces isolats sélectionnés ont été ensuite identifiés par séquençage de l'ADNr 16S comme étant *Lactococcus* (*Lc.*) *Lactis* et *Lactobacillus* (*Lb.*) *brevis*. D'autre part, l'étude de la composition de la poudre de la caroube en polyphénols a montré une teneur élevée en acides phénoliques et flavonoïdes présents sous forme libre, conjuguée ou liée, l'acide gallique et la rutine étant les plus abondants. Lorsque la poudre de caroube a été soumise à une digestion gastro-intestinale simulée, les polyphénols ont été libérés et sont devenus plus accessibles, ce qui a entraîné une augmentation de la capacité antioxydante. En combinant *Lc. lactis* C15, les deux probiotiques *Lb. brevis* B13 et B38 avec la poudre du caroube à 4%, un lait fermenté synbiotique a été développé avec succès. Les caractéristiques physicochimiques, la viabilité probiotique, la teneur en phénols totaux (TPC) et la capacité antioxydante ainsi que l'activité d'hypoglycémie du lait fermenté ont été déterminées pendant la conservation au froid (28 jours / 4°C) et la digestion gastro-intestinale. La supplémentation du lait fermente avec la poudre de la caroube a amélioré la croissance de *Lb. brevis* et a maintenu sa viabilité pendant la période de stockage (8 log CFU/g) et après la digestion gastro-intestinale (7 log CFU/g). Le lait fermenté a également montré une TPC et une capacité antioxydante élevées durant le stockage. La digestion *in vitro* du lait fermenté a entraîné la libération de composés phénoliques bio-accessibles où l'acide gallique (441%) et la (+) - catéchine (486%) étaient les composés phénoliques les plus quantifiés; ainsi, une forte inhibition des activités α-amylase (52%) et α-glucosidase (37%). Ces résultats démontrent le potentiel du lait fermenté a base de la caroube en tant qu'aliment fonctionnel pour être une source importante de probiotiques viables et de polyphénols bio-accessibles offrant des avantages pour la santé.

**Mots clés :** Lait fermenté, synbiose, *Lactococcus lactis*, *Lactobacillus brevis*, caroube, digestion gastro-intestinale, polyphénols, activité hypoglycémique



**ملخص**

في هذه الدراسة، تم تطوير حليب مخمر جديد باستخدام بكتيريا بروبيوتيك و مسحوق الخروب كمكون بريبيوتيك. تم عزل 118 سلالة بكتيرية تنتمي الى اجناس *Lactococcus* و*Lactobacillus* من الأجبان المحلية و الجزاءرية و تم التحقق من امكاناتها التخمرية و البروبيوتيك. اولا تم فحص جميع السلالات بحثا عن نشاطها المضاد للبكتيريا ضد اثنين من مسببات الأمراض المنقولة بالغذاء *Escherichia coli* و *Staphylococcus aureus* باستخدام تقنيات البقع و الآبار. بناءً على هذا الفحص ، تم اختيار 5 من المكورات اللبنية و 14 عزلة من العصيات اللبنية ودُرست علاوة على ذلك لصفاتها التكنولوجية والبروبيوتيك. أظهرت عزلة واحدة (C15) من المكورات اللبنية إمكانات تكنولوجية مثيرة للاهتمام مثل ارتفاع نسبة الحموضة في الحليب ، وأنشطة التحلل الدهني الجيدة والمحللة للبروتين. أظهر اثنان من العصيات اللبنية (B13 و B38) معدلات بقاء مشجعة تحت ظروف الجهاز الهضمي (8.20 ، 7.27 CFU / مل) بالإضافة إلى قدرة قوية على الالتصاق بخلايا HT-29 وقدرة عالية على مضادات الأكسدة و أنشطة تكنولوجية جيدة. تم التعرف على العزلات المختارة بواسطة تسلسل 16S rDNA على أنها *Lactococcus (Lc.) lactis* و *Lactobacillus (Lb) brevis*. من ناحية أخرى ، أظهرت دراسة تركيبة البوليفينول للخروب نسبة عالية من الأحماض الفينولية والفلافونويد الموجودة في أشكال حرة ومترابطة أومرتبطة مع وجود حمض الغال والروتين بوفرة. عندما تم تقديم مسحوق الخروب لمحاكاة الهضم المعدي المعوي ، تم إطلاق مادة البوليفينول وأصبح الوصول إليها أكثر سهولة مما أدى إلى زيادة قدرة مضادات الأكسدة. من خلال الجمعبين بين Lc. lactis C15 كلا البروبيوتيك Lb. brevis، مع مسحوق الخروب بنسبة 4٪ تم الحصول على حليب مخمر ناجح. تم تحديد الخصائص الفيزيائية والكيميائية ، وحيوية البروبيوتيك ، والمحتوى الفينولي والقدرة المضادة للأكسدة وكذلك نشاط نقص السكر في الدم للحليب المخمر الخروب أثناء التخزين البارد في 28 يوما/ 4 درجات مئوية وأثناء الهضم المعدي المعوي. أدى إضافة مسحوق الخروب إلى الحليب المخمر إلى تحسين نمو البروبيوتيك Lb. brevis والحفاظ على صلاحيتها خلال فترة التخزين (8 log CFU / g) وبعد الهضم (7 log CFU / g). أظهرأيضا الحليب المخمر محتوى إجماليًا من مادة البوليفينول وقدرة عالية من مضادات الأكسدة أثناء التخزين. أدى الهضم في المختبر للحليب المخمر إلى إطلاق مركبات فينولية يمكن الوصول إليها حيوياً حيث كان حمض الغال (441٪) و (+) - كاتشين (486٪) من أكثر المركبات الفينولية التي تم تحديدها كمياً مما أدى إلى تثبيط قوي لمضادات الأكسدة وأنشطة α-amylase (52٪) و α-glucosidase (37٪). توضح هذه النتائج إمكانات حليب الخروب المخمر كغذاء وظيفي ، والذي يمكن أن يكون مصدرًا مهمًا للبروبيوتيك الحيوي والبوليفينول الذي يمكن الوصول إليه بيولوجيًا ، مما يوفر فوائد صحية

**الكلمات المفتاحية:** الحليب المخمر ، السينبيوزيس ، لاكتوكوكوس لاكتيس ، لاكتوباسيلوس بريفيس ، الخروب ، الهضم المعدي المعوي ، البوليفينول ، نشاط مضادات الأكسدة ، نشاط سكر الدم