DEVELOPMENT OF PROCESSES FOR THE PRODUCTION OF POSTBIOTIC FUNCTIONAL FOODS

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Riassunto

Questo progetto - in collaborazione con la Kraft Heinz - ha lo scopo di sviluppare processi fermentativi per la produzione di componenti funzionali di tipo postbiotico. Il processo di fermentazione è stato sviluppato in scala laboratorio utilizzando due differenti substrati, latte scremato e sospensione acqua/farina di riso al 15%$_W$, e il microrganismo *Lactobacillus paracasei CBA L74* brevettato dalla Kraft Heinz.

Le attività di dottorato sono state condotte essenzialmente in due parti:

1. sviluppo di componenti funzionali postbiotici a scala di laboratorio;
2. sviluppo dei detti componenti a scala pilota.

Le attività condotte nella prima parte sono state le seguenti:

1.1. Sviluppo del protocollo di fermentazione per i due substrati.

La fermentazione è stata condotta in un reattore cilindrico incamiciato batch da 1,5 L. Dopo la sterilizzazione del sistema, il substrato viene caricato, inoculato con il microrganismo e lasciato fermentare per tempi necessari all'ottenimento della produttività desiderata.

La fermentazione è asservita da:

- sistema di miscelazione;
- sistema di misura e controllo della temperatura e del pH.

1.2. Caratterizzazione del fermentato e dei componenti funzionali prodotti.

Ottimizzato il processo su scala laboratorio, ci si è occupati di caratterizzare il fermentato ottenuto in termini di crescita batterica, produzione di acido lattico e produzione del metabolita funzionale. Attraverso studi condotti da altri partner di ricerca, è stato possibile identificare il componente funzionale e la sua sequenza. In questo lavoro di tesi, è stata messa a punto una metodica di analisi utilizzando la RP-HPLC per la sua quantificazione di routine.

1.3. Studio dell'effetto biologico dei metaboliti funzionali

Mediante l'ausilio di modelli cellulari di tipo Caco2 è stato condotto uno studio, in collaborazione con altri gruppi di ricerca, finalizzato a

identifcare i metaboliti prodotti durante la fermentazione del riso in grado di interferire con l'ingresso del peptide P31-43 (peptide della gliadina coinvolto nel processo infiammatorio intestinale nella malattia celiaca) all'interno delle cellule.

1.4. Sviluppo di semilavorati funzionali secchi a scala laboratorio mediante l'utilizzo di processi di Freeze-Drying

Al termine del processo di fermentazione i prodotti fermentati sono stati 'inattivati' tramite un blando trattamento termico per la riduzione della carica batterica, ed essicati mediante il processo di liofilizzazione.

Nella seconda parte della tesi l'attività è stata:

2.1. *Scale-up* del processo di produzione del semilavorato

La seconda parte del progetto ha riguardato lo *scale-up* del processo a livello pilota. L'impianto pilota dove sono state svolte le prove si trova nel centro ricerca e sviluppo 57 della Kraft Heinz a Nijmegen, Paesi Bassi.

Le prove sono state svolte in reattore batch da 300 L e sono state monitorate in termini di crescita batterica e produzione di acido lattico e metabolita attivo.

I risultati ottenuti nel corso della campagna di prove in scala laboratorio che in scala pilota hanno dimostrato buoni risultati in termini di crescita batterica, produzione di acido lattico e di metabolita attivo.

Al fine di verificare la stabilità del componente funzionale postbiotico ottenuto, sono state progettate prove di 'shelf life' a tre differenti temperature di conservazione e sulle differenti matrici fermentate.

Summary

This project - in collaboration with Kraft Heinz - aims to develop processes for the production of postbiotic functional components. The project is divided into several activities. The first part of the project regarded the development of a laboratory scale fermentation process. The strain used in the fermentation tests is *Lactobacillus paracasei* CBA L74 patented by Kraft Heinz. Fermentation tests were carried out on two different substrates: skimmed milk, and 15%w water/rice flour suspension. Fermentation is carried out in a 1.5 L batch reactor. The system is sterilized, loaded with the substrate, inoculated with the microorganism and left to ferment for established production times. Then, the product is removed from the reactor and the reactor prepared for a new cycle. The process is controlled in terms of:

- mixing;
- pH;
- temperature.

Once the process was optimized on a laboratory scale, the characterization of the obtained fermented broth was performed. The sequence of the functional component and its size were characterized by means of MALDI-TOF analysis. In addition, a RP-HPLC method was developed for its routine quantification. A study on Caco-2 cells, conducted in collaboration with other research groups, has demonstrated the positive effect of metabolites present in the supernatant of the fermented substrate, capable of interfering with the entry of the gliadin peptide P31-43 into the cells. These metabolites belong to a small active fraction (<10 kDa). Then, it was important to obtain a dry postbiotic. Studies have been carried out to choose the best method to shock/ kill the microorganism in the fermented broth, so as to obtain a stable product with a microbial load $<10^2$ CFU/mL. Subsequently, the dried product was obtained by freeze-drying.

The second part of the project regarded the 'scale up' of the pilot process. The pilot plant where the tests were carried out is located in the Kraft Heinz research and development centre 57 in Nijmegen, the Netherlands. These tests were carried out in a 300 L batch reactor and were monitored in terms of bacterial growth and production of lactic acid and active metabolite. The peroformance of laboratory scale and pilot scale fermentations were characterized by promising results in terms of bacterial growth, lactic acid production and active metabolite. In order to verify the stability of the obtained postbiotic functional component, 'shelf life' studies are being done at three different storage temperatures and on the different fermented matrices.

Introduction

There has been growing interest from consumers to better understand the food and its effects on the body. The traditional way of consuming food that ‘simply’ provides energy and alleviates hunger isn’t enough anymore. In addition, available information regarding the human microbiota, its composition and the profound impact it has on the human health, has pushed consumers towards a new class of food, known as functional foods.

Functional foods - or better, foods that have an impact on human health beyond the traditional nutritive values - have been the focus of science and industries alike. Togther, they have been working to provide food or food ingredients that satisfy the needs of consumers through the development of probiotics, prebiotics, synbiotics and most recently postbiotics.

Although the definition of functional food and the development of the various classes is ‘fairly new’, the consumption of such foods dates back to the beginning of the human civilization. Fermentation presents a natural method for the production of such foods. Records available provide evidence of the use of fermentation all around the world. The focus is on dairy based fermented foods - such as sour milk- and on cereal (rice, wheat, corn and sorghum) based fermented foods and beverages.[1]

The following work is divided in several chapters; in chapter I, functional foods (probiotics, prebiotics and postbiotics) are discussed, chapter II reports notions regarding fermentation, the different types, the microorganisms employed, the different substrates and the bioreactors. Chapter III discusses, the materials and methods used to achieve the goals set for this project, for both laboratory and pilot scale. Finally, results obtained are presented and discussed in chapter IV.

Aim

The main focus of this project is the development of an optimal process for the production of functional postbiotic components via lactic acid fermentation carried in batch reactor. This goal favors the growth of a probiotic bacteria in prebiotic substrates, during which active metabolite(s)-postbiotics- are produced.

In particular, this project aims to:

- Characterize the fermentation process of the selected strain under controlled conditions:
    - kinetics of the fermentation process;
    - characterization of metabolites;
    - selection of operating conditions to maximize yield and productivity of selected products;
- Develop a process to produce 'wet' functional products.
    - characterization of the fermentation process of the selected strain on natural based feedstock. The activity is also focused on the kinetics of production of primary, secondary and functional by-products;
    - optimization of the production process;
- Develop a process to produce 'dry' functional products
    - selection of the optimal technology to dry/shock/ kill the microorganisms;
    - analysis of intestine-metabolite interactions;
    - scale-up of the process at pilot scale.

Briefly, for each fermentation substrate used, whether milk or rice flour, two fermenting conditions are tested, with and without pH control, in order to investigate the effect of this parameter on the bacterial growth and lactic acid production. Then, the protocol with better results is adopted as our standard fermentation protocol and is scaled up to pilot plant. Furthermore, the functional properties of the postbiotic produced during rice flour fermentations are tested on Caco-2 cells.

The results obtained on Caco-2 cells using the cell free supernatant of rice flour and water suspension were promising. The results prompted other tests to verify the ability of our strain to ferment on minimal matrices. This condition reduces feed preparation costs and allows energy savings as the viscosity of such matrices is similar to water. It should also facilitate the extraction of the active metabolites and their analysis.

Chapter I

Functional foods

The concept of functional foods was first promoted in Japan in 1980s by scientists studying the relationships between nutrition, sensory satisfaction, fortification and modulation of physiological systems [2]–[4]. The term 'functional foods' refers to a variety of foods that promote health in ways not anticipated by traditional nutritional science [5]. Such foods impact the human health and induce one or more physiological function increasing health and/or decreasing the risk of diseases [6].

The variety of functional foods that can be developed is unlimited thus creating a lot of challenges for scientists and industries alike, to study the effect of such modifications on human health and find a suitable application.

1.1 Probiotics, prebiotics and synbiotics

Probiotics are defined as living microorganisms which, when ingested in certain amounts, have a positive impact on host's health that goes beyond conventional nutritional effects [7]–[9]. Table (1) reports current microorganisms used as probiotics [10].

Table 1: Current microorganisms used as probiotics [10]

Current microorganisms used as probiotics		
Sl. No.	Probiotic bacterial genera	Species involved
1	Lactobacillus	L. plantarum, L. paracasei, L. acidophilus, L. casei, L. rhamnosus, L. crispatus, L. gasseri, L. reuteri, L. bulgaricus
2	Propionibacterium	P. jensenii, P. freudenreichii
3	Peptostreptococcus	P. productus
4	Bacillus	B. coagulans, B. subtilis, B. laterosporus
5	Lactococcus	L. lactis, L. reuteri, L. rhamnosus, L. casei, L. acidophilus, L. curvatus, L. plantarum
6	Enterococcus	E. faecium
7	Pedicoccus	P. acidilactici, P.pentosaceus
8	Streptococcus	S. sanguis, S. oralis, S. mitis, S. thermophilus, S. salivarius
9	Bifidobacterium	B. Longum, B. catenulatum, B. breve, B. animalis, B. bifidum
10	Bacteroides	B. uniformis
11	Akkermansia	A. muciniphila
B	Saccharomyces	S. boulardii

As seen in table (1), bacteria constitutes a large part of probiotics especially the genera *Lactobacillus* and *Bifidobacterium*. To the date, probiotic bacteria are mostly consumed in dairy based foods such as yoghurt or in freeze-dried form. Once the probiotic bacteria reaches the intestinal tract, in particular the colon, it can establish transiently in the large bowel and induce changes in the gut microflora.

In addition to enhancing the gut health, several studies have demonstrated the effect probiotics have on other physiological

functions such as: alleviation of lactose intolerance [11], immune enhancement [12]–[14], reduction of dermatitis symptoms [15], antioxidative effects [16], prevention of cancer [17], reduction of mineral absorption [18], cholesterol lowering effects [19] and antihypertensive effects [20].

A number of possible mechanisms of action have been proposed in an attempt to explain how probiotics induce such beneficial effects. These may be mediated by a direct antagonistic effect against specific groups of microorgainsms, thus decreasing their numbers or affecting their metabolism (e.g. production of antimicrobial substances, competition for nutrients, competition exclusion of pathogens) or by stimulation of the immune system (e.g. assist in early programming of the immune system) [21]–[24].

Foods supplemented with probiotics are usually regarded as safe, and they must contain probiotic organisms in sufficient numbers at the time of consumption in order to exert their effect [25]. However, the viability of live bacteria in food products and during transit through the gastrointestinal tract may be variable. To limit the effects connected to the reduction of the bacteria viability, the prebiotic concept has been developed.

Prebiotics are short chain carbohydrates that are not digestible by digestive enzymes in humans and selectively enhance the activity of some groups of beneficial bacteria [26], [27]. The chemical nature of prebiotics inhibit their digestion by the pancreatic and small-bowel enzymes in the human gut, therefore they reach the large bowel.

They include fibres, fructooligosaccharides (FOS), galactooligosaccharides (GOS) inulin and lactulose [26]. Table (2) includes a list of types of prebiotics and their sources. [27]

Table 2: types of prebiotics and their sources (adapted [27]*)*

Type of prebiotic	Source of prebiotic
Fructooligosaccharides	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichole, wheat, honey, banana, barley, tomato and rye
Isomaltulose	Honey, sugarcane juice
Galactooligosaccasirides	Human's milk and cow's milk
Cyclodextrins	Water-soluble glucans
Enzyme-resistant dextrin	Potato starch
Arabinoxylooligosaccharides	Wheat bran
Maltooligosaccharides	Starch
Palatinose	Sucrose
Lactosucrose	Sucrose
Soybean oligosaccharide	Soybean
Raffinose oligosaccharides	Seeds of legumes, lentils, peas, beans, chickpeas, mallow composite and mustard
Xylooligosaccharides	Bamboo shoots, fruits, vegetables, milk, honey and white bran.

As reported in table (2), prebiotics are found in a number of sources (e.g. fruits, vegtables, legumes) that are part of the human diet.

In order to be classified as prebiotics, food should fulfill the following criteria [28]:

- ✓ It must not be hydrolysed and/ or absorbed in the upper part of the gastrointestinal tract;
- ✓ It must address the composition of the colonic microbiota towards a healthier composition;
- ✓ It must induce effects that are beneficial to the host's health.

Prebiotics like inulin and pectin exhibit several health benefits, for example the reduction of prevalence and duration of diarrhea, relief from inflammation and other symptoms associated with intestinal bowel disorder and protective effects against colon cancer [29]. Furthermore, a study by Ley et al.(2006), linked prebiotic consumption in diets to reducing the risk of obesity [30].

Prebiotic intake, especially fructans, has been associated with enhancement of calcium absorption [31], while the addition of galactooligosaccharides to the diet of rats increased the absorption of both calcium and magnesium [32].

A further possibility to enhance the composition of the colonic (gut) microbiota is the use of probiotics in combination with prebiotics. This combination is known as synbiotics. The result should be an improved survival of the probiotic, which has an immediately available substrate for its fermentation.

A study by Gmeiner et al. (2000), showed that the microbial community of the gastrointestinal tract exists in a dynamic state, and can be affected by the introduction of synbiotics (*L. acidophilus* with

fructooligosaccharides) [33]. Ashara et al. (2001) [34] demonstrated that the introduction of bifidobacteria together with transgalactooligosaccharides (TOS) during antibiotic treatment may be used against opportunistic intestinal infections with antibiotic-resistant pathogens. Malaguarnera et al. (2011) [35]conducted a study to determine the effects of synbiotic on inflammation. Two groups were studied, the first receiving synbiotic (*Bifidobacterium longum* with fructooligosaccharides) and life style modifications (e.g. diet and excersice) while the second group had lifestyle modification alone. Results showed that the introduction of a synbiotic group reduced the levels of tumor necrosis factor (TNF)-α and C- reactive protein (CRP) both involved in inflammation.

Despite the many benefits of probiotic intake - and the history of safe use - introducing live microorganisms in the human body could be a potential risk especially in immunocompromised and genetically predisposed individuals [36]. This has been a driving factor for the rise of a new approach: the direct use of beneficial probiotic derived factors without the use of live bacteria.

1.2 Postbiotics

Probiotic derived factors - also known as postbiotics - can optimize various physiological functions of the host, offering the additional advantage of the ability to use them even in immunodepressed individuals [36].

These factors, able to induce the beneficial effect, can either be microbial metabolites (e.g. enzymes, proteins, peptides, polysaccharides, organic acids and lipids) [37]–[43] or microbial components (e.g. lipoteichoic acids, teichoic acids, peptidoglycan, polysaccharides and cell-surface porteins) [44], [45].

The identification of such factors represents a new opportunity to develop new therapeutic strategies, in which the administration of live bacteria could be easily avoided [46]. In fact postbiotics are being used either alone or in combination with prebiotics as a treatment for microbiota dysbiosis [47].

A number of studies has been conducted either *in vitro* (e.g. cell lines) or *in vivo* (e.g. mice and rats) to assess the potential effect or bioactivity of various postbiotics. *L. acidophilus ATCC 4356* cell free supernatant contained postbiotic molecules of a protein nature, that were able to inhibt the growth of several strains of *Campylobacter jejuni* [41]. Postbiotic molecules produced by different lactobacilli strains (*L. Johnsonii* La1, *L. rhamnosus* GG, *L. casei Shirota* YIT9029, *L. casei* DN-114001 and *L. rhamnosus* GR1) caused a decrease in the viability

of *Salmonella enterica* [48]–[50]. The growth of pathogenic bacteria such as *Escherichia coli*, *Salmonella* and *Shigella* was inhibited due to the activity of postbiotic molecules produced by different strains of Lactobacilli and Bifidobacterium [51].

Postbiotics are also involved in the maintenance of cellular integrity; *L. rhamnosus* GG produce a molecule involved in the suppression of alcohol-induced increased intestinal permeability and endotoxemia [52]. Furthermore, supplementation with postbiotics reduces blood pressure, conferring antihypertensive capacity to these compounds [53].

The activity of various postbiotics depends on their nature, medium's pH, and the various treatments of the growth medium such as heat treatment and /or enzymatic treatment [54].

Different methods can be used to extract and separate postbiotic molecules, these include heat-treatment [44] and sonication [55]. Moreover, different analytical methods such as liquid chromatography (HPLC), matrix assisted laser desorption/ ionization time of flight (MALDI-TOF) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR) can be implied.

1.2.1 *Bioactive peptides*

Bioactive peptides are a class of postbiotics and are considered the new generation of biologically active regulators. Their production could take place during the manufacture of fermented foods. Usually they are low in molecular weight and contain from 2-20 amino acids joined by covalent bonds [56]. The amino-acid composition and sequence play a role in their activity. Biological activities that have been attributed to bioactive peptides include antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulatory, mineral binding and anti - oxidative effects [57].

There are more than 1500 classified bioactive peptides. They derive from various animal sources (bovine milk, cheese, eggs and meat) and vegetal sources such as soybeans, cereal grains (wheat, barley, rice, rye, oat, millet, sorghum and corn) [56].

Chapter II

Fermentation

Records of fermentation to preserve foods go back to the beginning of human civilization. Fermentation of meats, vegetables and cereals date back to 6000 BC [1]. Fermentation is a metabolic process that produces chemical changes in organic substrates through the action of enzymes. More precisely, in the context of food production, it may more broadly refer to any process in which the activity of microorganisms causes desirable change to the foodstuff or beverage. In fact, fermentation provides a natural way to destroy undesirable components, to enhance the nutritive value and appearance of food and reduce the energy required for cooking [58].

For microorganisms, fermentation is the primary means for producing ATP by the degradation of organic nutrients anaerobically. Four main fermentation processes have been identified [1].

- Alcoholic fermentation: also known as ethanol fermentation. This process converts sugars (glucose, fructose and sucrose) into cellular energy producing ethanol and CO_2 as by-products. The main microorganisms used are yeasts, mostly *Saccharomyces cerevisiae* and *Schizosaccharomyces*.
- Acetic acid fermentation: in the presence of excess oxygen, bacterial strains of the genera *Acetobacter* and *Acetomonas* covert alcohol into acetic acid.
- Alkali fermentation: during which proteins of raw materials are broken down into amino acids and raising the final pH of the food. This kind of fermentation is achieved mostly spontaneously by mixed bacteria cultures, mostly dominated by *Bacillus subtilis.*
- Lactic acid fermentation which is described in details below.

2.1 Lactic acid fermentation

Lactic acid fermentation is a metabolic process by which glucose and typically other six carbon sugars (oligosaccharides of six carbons include sucrose and lactose) are converted into cellular energy and the metabolite is lactate (dissolved lactic acid). This fermentation is carried out under anaerobic conditions and occurs in some bacteria and some animal cells such as muscle cells. Facultative anaerobic bacteria will both ferment and undergo respiration in the presence of oxygen.

Lactic acid is the most widely occurring carboxylic acid in nature, and can be manufactured by chemical synthesis and by carbohydrate fermentation. Figure (1) reports the pathway of lactic acid production through fermentation [59].

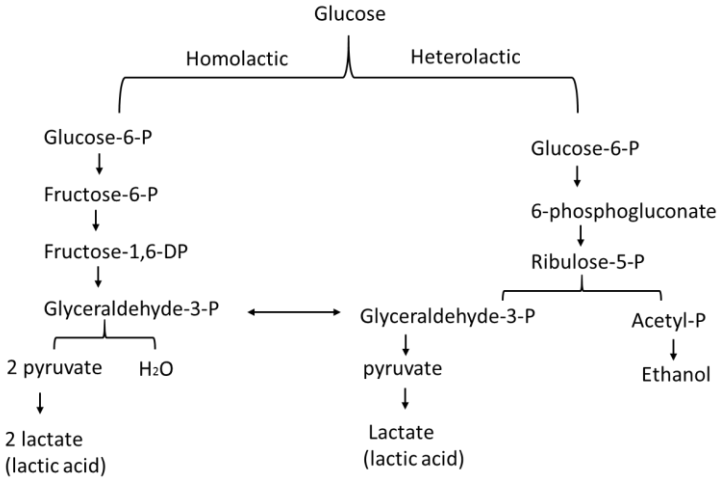


Figure 1: Lactic acid production through fermentation. Glucose is converted to lactate (lactic acid) through two different routes: homolactic in which lactic acid is the only metabolite and heterolactic in which ethanol is produced as well as lactic acid (adapted by [59]*).*

Homolactic fermentation is the simplest type of fermentation. Pyruvate produced by glycolysis of glucose undergoes a redox reaction forming lactic acid. This fermentation does not produce gas as a by-product. One molecule of glucose (6 atoms of carbon) is converted into 2 molecules of lactic acid (3 atoms of carbon) as shown in figure (2)

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$$

Figure 2: Conversion of one molecule of glucose into 2 molecules of lactic acid

This fermentation occurs for example in milk to produce yoghurt, where the lactic acid produced gives the yoghurt its characteristic sour taste.

The second pathway for lactic acid production is heterolactic fermentations in which other products (such as ethanol or CO_2) are produced along with lactic acid. This type of fermentation is performed by lactic acid bacteria or LAB.

2.1.1 Lactic acid bacteria (LAB)

The concept of LAB as a group of microorganisms was developed in the beginning of 1900s thanks to previous work of scientists such as Pasteur who made significant contributions to lactic acid fermentation in 1857, and Lister, who in 1873, isolated for the first time a pure

bacterial culture. Furthermore, the introduction of the use of starter cultures for the production of cheese and sour milk happened almost immediately by Weigmann in Kiel and Strorch in Copenhagen in 1980. This allowed the industrialisation of food fermentations through the use of LAB.

LAB are typically Gram-positive, non-spore-forming rods or cocci. They produce lactic acid as the main end product of glucose (carbohydrates) fermentation and share some common metabolic and physiological characteristics [60]. Table (3) reports their classification according to Orla-Jensen (1919) into 7 families, different in shape and kind of fermentation [61]. LAB are generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also members of the flora of the mouth, intestine and vagina of mammals [62].

Table 3: Lactic acid bacteria (adapted by [61]*)*

Genus	Shape	Fermentation	Current genera
Betabacterium	Rod	Hetero-	Lactobacillus Weissella
Thermobacterium	Rod	Homo-	Lactobacillus
Streptobacterium	Rod	Homo-	Lactobacillus
Streptococcus	Coccus	Homo-	Streptococcus Enterococcus Lactococcus Vagococcus
Betacoccus	Coccus	Hetero-	Leuconostoc Cenococcus Weisselle
Microbacterium	Rod	Homo-	Brochothrix
Tetracoccus	Coccus	Homo-	Pedicoccus Tetragenococcus

Lactobacillus is the largest genus within the group of LAB. Based on the 16S rRNA sequences, lactobacilli are phylogenetically distributed in seven groups:

1. *L. buchneri* group (bu);
2. *L. casei* group (ca);
3. *L. delbrueckii* group (de);
4. *L. plantarum* group (pl);

5. *L. reuteri* group (re);
6. *L. sakei* group (sa);
7. *L. salivarius* group (sl) [61].

The genus is split into three groups based on the carbohydrate fermentation pathways:

- Obligate homofermentative
- Facultative heterofermentative
- Obligate heterofermentative

Species that belong to the obligate homofermentative group ferment almost exclusively through the glycolytic pathway and are unable to ferment the pentoses and gluconate. Facultative heterofermentative bacteria ferment the hexoses through glycolysis with the production of lactic acid behaving as homofermentative bacteria. In presence of pentoses, they oxidize these compounds via the pentose phosphate route, without producing CO_2. Obligate heterofermentative species can not degrade glucose via glycolysis because they lack fructose 1,6 diphosphate aldolase that catalyzes the cleavage of fructose 1,6 diphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. For this reason, they ferment glucose through the pentose phosphate pathway, obtaining three final products in equi-molar ratio: lactic acid, ethanol and CO_2.

Foods that undergo lactic fermentation using LAB may present changes in chemical-physical, organoleptic, sensorial and nutritional characteristics, sometimes even improved, caused by the activity of the microorganisms. These fermented foods also have longer shelf life and sometimes even a higher degree of safety of use. In fact, the industrial importance of LAB is evidenced by their status as GRAS (generally regarded as safe) due to their ubiquitous appearance in food and their contribution to the health of microbiota of animals and human. LAB can ferment a variety of substrates including milk and cereals.

2.2 *Fermentation feedstocks*

People have fermented milk, cereals (e.g. rice, corn, sorghum, wheat, millet, soybeans), legumes (e.g. beans), fish and meat. Fermentation of each feedstock introduces changes to the original feedstock.

Milk

Milk is the characterizing excretion of mammals, produced to meet the complete nutritional and some defensive and other physiological requirements of the neonate of the species. Humans have used the milk of other species in their diets for thousands of years. In various parts of

the world, fermentation of milk - referred to as sour milk - was done at home to preserve the milk from spoilage. Nowadays, a major industry has been developed around milk considering its flexibility and the wide range of possible products. The milk is an aqueous solution of lactose, inorganic and organic salts, and numerous compounds at trace levels. It contains approximately 3.5% proteins of which 80% are casein and 20% whey proteins. Caseins have been classified as α, β and k-caseins. Whey contains β-lactoglobulin, α-lactalbumin and several minor proteins.

The milk contains lactose which is an allergen. Lactose intolerance is the most common disorder of carbohydrate absorption leading to diarrhea. Lactase deficiency occurs in the majority of human adults throughout the world and appears to be genetically determined. Fermentation of milk decreases lactose quantities thus protecting against such intolerance [63].

In general, the technology for milk fermentation is relatively simple and cost-effective. Standardized fermented milk products are manufactured in large scale production and are widely available on the market for consumers.

Some types of fermented milk are characterized by specific culture organisms used for fermentation: yogurt must be fermented by the symbiotic relationship of *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus*. It can also be fermented by *S. thermophilus* in combination with any *lactobacillus* species. Acidophilus milk shall be fermented by adding a culture of *L. acidophilus*. Kefir shall be fermented using a starter culture prepared from kefir grains, *Lactobacillus* and *Acetobacter* [64]. Most types of fermented milk are made from cow's, buffalo's, sheep's and goat's milk [65].

Fermentation of milk by LAB is an attractive approach to generate functional foods enriched with bioactive peptides. Studies show that consumption of fermented milk containing bioactive peptides has beneficial effects such as blood pressure lowering effect in hypertensive subjects [66].

Rice

Today, most of the fermented products found on the market are dairy based, but growing demand for functional foods pushed the industries towards new ingredients for their formulation. Cereals are an optimal choice to produce functional foods due to their abundance and nutritional characteristics. In fact, cereals cover 73% of the total cultivated areas of the world and contribute to 60% of the world's

population food, providing carbohydrates, proteins, fiber, energy, minerals and vitamins useful for human health [67]. The possible applications of cereals or their constituents in the formulation of functional foods are:

- ✓ Fermentable substrates for the growth of probiotic microorganism;
- ✓ Fibers promoting various beneficial effect;
- ✓ Prebiotics for the presence of specific non digestible carbohydrates.

Rice is one of the most consumed cereals in the world and represents a staple food for more than half of the world's population [68]. It belongs to the grass family, genus *Oryza, sativa* species. *Oryza sativa* is divided into three subspecies: *indica, japonica* and *javanica*. *Javanica* is not widespread, while the other two are grown all over the world.

Like all cereals, rice is characterized by a fruit called grain or caryopsis.The grain is an indehiscent dry fruit, that is unable to open and release the seed contained inside. It is possible to divide the kernels into three main parts:

- ✓ An external area, that is a series of layers that surround the seed and make up the part called bran;
- ✓ An internal part, called the endosperm;
- ✓ The germ that represents the embryo of the new plant.

The external part consists mainly of vegetable fibers, proteins, minerals salts and vitamins. It is undoubtedly the richest part of nutrients useful for the body. The endosperm is mainly made up of starch, few minerals and few vitamins. The germ is rich in vitamins, salts, minerals, proteins and is the only part of the caryopsis that contains lipids. Rice is the lowest protein cereal (7.5%) however the nutritional quality is higher than that of wheat proteins. It is the richest in glycides in the form of starch. It is one of the most digestible and assimilable plant foods because it contains very little cellulose, but contains vitamin B in particular B1.

Starch is the most abundant component of the caryopsis and represents the main energy source for humans. It is a polysaccharide made up of two different molecular complexes: amylose 20% and amylopectin 80% [69].

Amylose is a water-soluble linear chain polymer; it can contain more than a thousand D-glucose units bound by 1-4 α-glycosidic bonds. By virtue of these bonds, the molecule assumes a helical structure. Amylose represents the amorphous part of the starch.

Amylopectin is a branched chain insoluble polymer and can contain up to five thousand D-glucose units bound by 1,4 α-glycosidic bonds. Every 20-30 residues there are branches in which units are linked by means of 1,6 α-glycosidic bonds. By virtue of these bonds, the structure is globular and finely spongy. It constitutes the semi-crystalline part of the starch. The structural and functional properties of the starch granules will depend on the organization of amylose and amylopectin inside the granules [67]. Made up of small polyhedral granules of 4-6 microns with point hilum, rice starch is more digestible than other starches. In fact, in healthy subjects, while 5-10% of the starch contained in wheat, potatoes and corn is not absorbed and undergoes intestinal fermentative processes by colon bacteria with production of hydrogen, rice starch is almost entirely absorbed.

Rice starch is a poly-enzymatic complex of plant origin particularly rich in digestive enzymes. These include amylases, lactases, proteases, acylases, lipases etc. Amylase allows digestion of starch in dextrins, maltose and isomaltose, lactase cleaves lactose disaccharide in glucose and galactose monosaccharides, proteases allows the digestion of proteins in amino acids, the lipases split the lipids in glycerol and fatty acids, the cellulase allows the splitting of cellulose into glucose.

2.3 Bioreactors

Bioreactors are reactors in which a process of chemical transformation takes place by microorganisms or their derived active substances. Usually the system is composed of: a vessel, an agitation system, a system for temperature control, a supporting system for different measuring instrument (for example: pH-meter) and a control system. When the process that is taking place inside the reactor is a fermentation process, the bioreactor is referred to as ‘fermenter’.

Fermenters vary in complexity from simple non agitated to ones with complex agitation and control systems. Regardless of their complexity, all fermenters are addressed to optimize the growth of the microorganism and/or favor the production of certain microbial product(s).

A spectrum of parameters should be measured and controlled by all fermenters:

- Sterility: the reactor should be sterile before inoculation of the microorganism. This is necessary to eliminate contaminations during the process.

- Temperature control: the optimal temperature for microbial growth varies, it is then necessary to have a system that controls and maintains efficiently the temperature throughout the fermentation.
- Homogeneity: feedstock, microorganism and products should be distributed equally inside the reactor to avoid process inhibition.

There are four different typology of bioreactor in the biochemical industry and are divided into agitated and not agitated systems.

1. Batch: Discontinuous closed system. It is the simplest from the design point of view, this allows a high variety of volumes, agitation ad control system to be implemented. The production cycle is expected to last for a certain time upstream and downstream for which there will be dead times necessary for sterilization, pre-treatment loading and unloading. The presence of downtime, from an industrial point of view, is certainly a limit, however the level of sterilization that is achieved with this type of system is preferable. It achieves high levels of substrate conversion. The variety of composition inside the reactor over time leads to control and optimization problems making it difficult to remain in optimal conditions.
2. Continuous: In the continuous bioreactor the dead times, compared to the batch reactor, are reduced. It is possible to work in steady state conditions and this entails greater efficiency both in terms of control and in terms of operating conditions. The disadvantages concern the high investment costs, they are unsuitable for high concentration levels and for low growth rates. In addition, the long duration of the cycle production can cause contamination problems. These reactors are not very versatile.
3. Fed-batch: The Fed-batch bioreactor combines some advantages and disadvantages of batch and continuous reactors. It allows rather long production cycles, while maintaining some operating variables constant since it provides continuous substrate input and absence of output currents. The main objective of this reactor is to optimize the input flow rate.
4. Plug-flow: The ideal Plug Flow Reactor (PFR) is a continuous tubular reactor model. Unlike the agitated reactors, however, it is a perfectly segregated reactor model, therefore inside there are composition gradients between input and output; this means that in each section of the reactor there is a variation in the composition of the system with respect to the previous section and the subsequent one. Therefore, the composition of the fluid is homogeneous in each section of the reactor, while it varies continuously along its main development

coordinate. This type of reactor isn't good to obtain an excellent fermentation process.

Chapter III
Materials & Methods

This chapter reports the materials and methods used in both laboratory and pilot scales, and the analytical methods used. The statistical and mathematical models used are also described.

More specifically, the strain, culture mediums, fermentation feedstocks chosen to perform the fermentations, the apparatus, fermenting protocols, analytical and experimental methods for each scale are reported.

Laboratory Scale

3.1 Materials at laboratory scale

3.1.1 Strain

Lactobacillus paracasei CBA-L74 (international depository accession number LMG-P24778) provided by Kraft Heinz Italia s.p.a was selected for the fermentation processes. The strain is a facultative anaerobe, mesophile with an optimal growth temperature of 37°C. It is conserved at -26°C in cryovials with glycerol (20%).

3.1.2 Fermentation feedstock

The feedstocks chosen to perform the fermentations were:

- Milk: at laboratory scale the milk used is UHT skimmed that is usually found on the market. Table (4) reports the nutritional values of the milk used for 100 mL:

Table 4: Nutritional information of skimmed milk per 100 mL.

Nutritional information per 100 mL	
Total fat	0.1 g
- Of which saturated fatty acids	0.01 g
Carbohydrates	4.8 g
- Of which sugars	4.8 g
Protein	3.2 g
Salt	0.1 g
Calcium	120 mg

- Rice flour: the rice flour used was provided by the KraftHeinz company. The nutritional values for rice flour unenriched, white per 100 g are reported in table (5).

Table 5: Nutritional information of white unenriched rice flour per 100 g

Nutritional information per 100 g	
Total fat	1.4 g
- Of which saturated	0.4 g
Total carbohydrates	80 g
- Of which dietary fiber	2.4 g
- Of which sugar	0.1 g
Protein	6 g
Calcium	10.00 mg
Potassium	76 mg
Iron	0.35 mg

- Rice flour supernatant: another feedstock used for fermentations is rice flour supernatant obtained from the centrifugation of rice flour and water suspension at two differnet centrifugal speeds. For these feedstocks, total proteins and glucose content were measured as reported in table (6) for the supernatant obtained at the first centrifugal speed and in table (7) for the supernatant obtained at the second centrifugal speed:

Table 6: Nutritional values for 100 mL of supernatant obtained at the first centrifugal speed

Nutritional information per 100 mL	
Glucose	0.21 g
Total proteins	0.038 g

Table 7: Nutritional values for 100 mL of supernatant obtained at the second centrifugal speed

Nutritional information per 100 mL	
Glucose	0.203 g
Total proteins	0.0085 g

3.1.3 Fermentation recipes

- *Milk*

At laboratory scale the recipe for milk fermentations is reported in table (8). Glucose (2%) was added to skimmed liquid milk (98%) usually found in the market.

Table 8: Milk fermentation's recipe

Ingredient	Percentage
Milk	98%
Glucose	2%

- *Rice flour and supernatant*

The recipe used to perform these fermentations is reported in table (9). The same recipe was also used to prepare the initial suspension for rice flour supernatant's fermentations.

Table 9: Rice flour fermentation's recipe

Ingredient	Percentage
Rice flour	15%
Glucose	1%
Amylase	0.015%
Water	84%

3.1.4 Animal free broth

Animal Free Broth (BAF) is used as a growth medium for *L. paracasei*, it is prepared following a precise recipe, sterilized in autoclave (122°C/30 min):

- In a Pyrex bottle
  1. Measure 100 mL of H_2O
  2. Weigh 10 g of glucose
  3. Add glucose to the bottle
  4. Mix until completely dissolved
- In another Pyrex bottle
  1. Measure 100 mL of H_2O
  2. Weigh 0.1 g of citric acid
  3. Weigh 0.1 g of magnesium sulfate
  4. Weigh 4 g of yeast extract
  5. Mix with water until completely dissolved
- Autoclave
- Wait until both mixtures cool down
- Add mixtures under flow laminar hood

3.1.5 Medium cultures

To evaluate the growth of Lactobacillus and other possible contaminants, the spread plate (A.1) method was used. This method requires the use of medium cultures that favor the growth of specific bacteria. Three mediums with different composition and selectivity were used:

- MRS (De Man, Rogosa and Sharpe)
  This medium is used to evaluate the growth of Lactobacillus. Its composition is reported in the table (10) below. Meat extract and peptone represent the source of carbon, nitrogen and vitamins for the growth. Yeast extract contains the vitamins and amino acids necessary specifically for the growth of Lactobacillus.

Table 10: Composition of MRS agar

Casein peptone	10 g
Meat extract	8 g
Yeast extract	4 g
Glucose	20 g
Dipotassium phosphate	2 g
Tween 80	1 g
Diamonium hydrogen citrate	2 g
Sodium acetate	5 g
Magnesium sulphate	0.2 g
Manganese sulphate	0.04 g
Agar	14 g

- Gelisate agar
  This medium is used to detect the presence of aerobic bacteria. It is transparent and its composition is reported in the table (11) below:

Table 11: Composition of Gelisate agar

Gelatin peptone	5g
Agar	15g

- MacConkey agar
  This medium is selective for Gram negative bacteria. It contains bile salts and crystal violet that inhibit the growth of gram positive bacteria. Its composition is reported in the table (12) below:

Table 12: Composition MacConkey agar

Agar peptone	17 g
Casein peptone	1,5 g
Meat peptone	1,5 g
Sodium chloride	5 g
Lactose	10g
Mix of bile salts	1.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar-agar	13,5 g

3.1.6 Cell cultures

Caco-2 cells were grown in DMEM (GIBCO, San Giuliano Milanese, Italy), 10% foetal bovine serum (FBS) (GIBCO) and 1 mM glutamine in an incubator at a temperature of 37°C.

3.1.7 Gliadin peptides

The synthetic peptides P31-43, used for Caco-2 cells stimulation (InBios, 95% pure, analysed by MALDI-tof) (A.2) were obtained through filtration of Ultrasat-D20 (Sartorius AG, Goettingen, Germany) the sequence of P31-43 is: LGQQQPFPPQQPPY.

The peptides were labelled with lissaminate (wave length 575/595) and were produced in the same way as non lissaminated peptides. Dextran-40-Tex-Red was used for fluorescence essay (Sondaggi Molecolari, San Giuliano Milanese, Italy)

3.2 Instruments at lab scale

3.2.1 Fermentation apparatus

The fermentation tests were carried in a closed sterile system consisting in a batch reactor, a mixing system, a thermostatic system, and a pH/ temperature control system.

The batch reactor has a total volume of 1.5 L, it is a cylindrical Pyrex (20cm high, 10cm ID) equipped with a jacket for the circulation of the thermostatic fluid.

The mixing system is a stainless steel impeller equipped with two different turbines: an inclined blade turbine and Rushton turbine that are used to provide axial and radial movement to guarantee homogeneity of the matrix. The high performance electrical engine coupled with the impeller system provided uniform mixing even with high density/viscosity broth.

pH and temperature are measured simultaneously using InPro3100i Mettler Toledo probe (A.3), with a diameter of 12 cm and a length of 15 cm.

Figure (3) shows the main part of the fermenter.

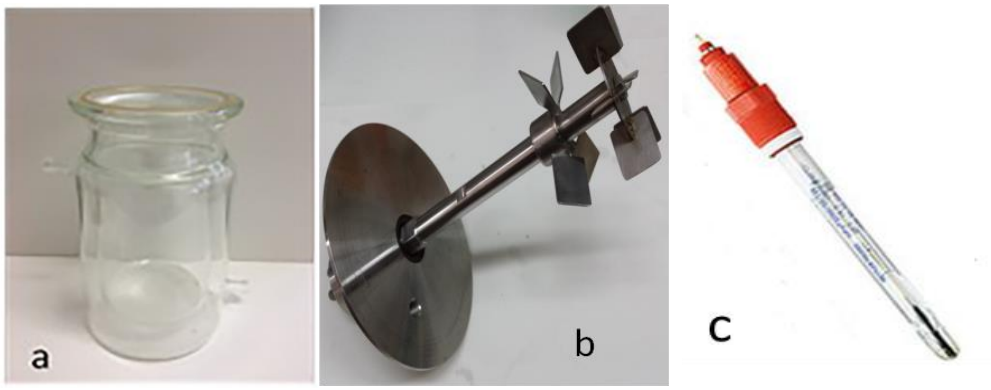


Figure 3: Main parts of the fermenter; (a) Pyrex vessel (b) impeller with Rushton turbine and inclined turbine (c) probe used to measure pH and temperature.

Figure (4) shows the assembled system.

Figure 4: The assembled system

The fermentations using rice flour supernatant were performed in sterile system that was previously described (figure 3a, 3c) with one difference regarding the mixing system; it consisted in a stainless-steel impeller equipped with two Rushton turbines as shown in figure (5):

- Impeller #1 has a diameter of 5 cm, with height/ width/ thickness of blades of 1.2/1.7/0.1 cm respectively.
- Impeller #2 has a diameter of 4 cm, with height/ width/ thickness of blades of 0.7/1/0.1 cm respectively.
  This mixing system was used to guarantee homogeneity of the matrix and avoid gradient temperature and microorganism concentration.

Figure 5: Modified impeller with two Rushton turbines used for supernatant fermentations

3.2.2 Drying system

To obtain dry powder of the fermented product freeze drying was used (A.4). The apparatus used is a bench freeze-drier Alpha 1-2 LD plus (Figure 6). Samples processed by the freeze dryer were heat treated at (90°C/1 min) to reduce the microbial charge and frozen at -26°C for at least 24h before starting the process that lasted for 24h (12h main drying and 12h final drying).

Figure 6: Freeze-dryer

3.3 Experimental methods

Each fermentation performed at a lab scale takes 48 hours between preparations and the process itself. Some steps are in common between the substrates, others differ due to the difference in substrates' nature. All steps are performed under laminar flow hood unless mentioned otherwise. All sterilization cycles in autoclave occur at 121°C for 20 minutes (also referred to as standard cycle) unless mentioned otherwise. Reported below are the steps necessary to perform the fermentations:

3.3.1 Strain propagation

The preparation of the strain takes place 24h before the fermentation process itself. 1 mL of strain/ BAF/ glycerol solution that is usually conserved at -26°C, is thawed under laminar flow hood, and added to 9 mL of BAF at room temperature. It is then incubated for 24h at 37°C.

3.3.2 Milk fermentations' protocol

The following figure (7) is a schematic representation of the steps of milk fermentations:

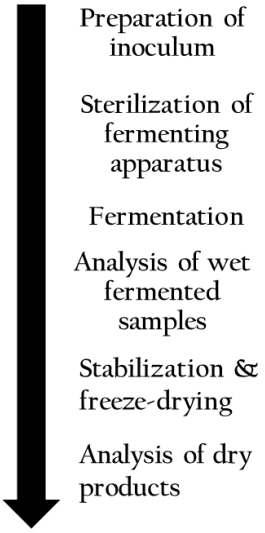


Figure 7: Schematic representation of milk fermentations

1. Reactivation of *L. paracasei* (incubation at 37°C for 24 h in BAF);
2. Sterilization of impeller, batch reactor, and pH/temperature probe in autoclave;
3. Preparation of NaOH and sterilization in autoclave; *
4. Glucose is dissolved in 40 mL of water and sterilized in autoclave;
5. Assembly of fermenter under laminar hood;
6. The fermenter is linked to the water bath and peristaltic pump;

7. Adjustment of temperature and pH;
8. Inoculation;
9. Manual sampling every two hours;
10. After 24 h end of fermentation
11. Analysis
12. Stabilization (heat treatment 90°C 1 min)
13. Freeze –drying (A.4)
14. Analysis of dried samples.

* only for pH controlled fermentations

Steps 12-14 are performed for the protocol that gave the best results in terms of bacterial growth and lactic acid production.

3.3.3 Rice flour fermentations' protocol

The following figure (8) is a schematic representation of the steps for rice flour fermentations:

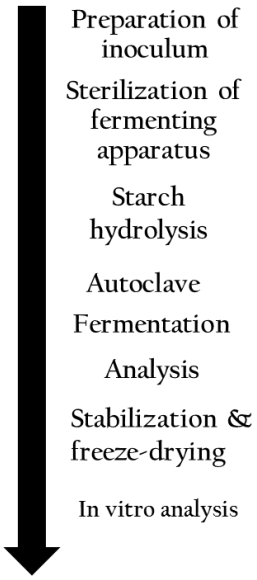


Figure 8: Schematic representation of rice flour fermentations

1. Reactivation of *L. paracasei* through incubation at 37°C for 24 h in BAF.;
2. Sterilization of impeller, batch reactor and pH/temperature probe in autoclave;
3. Preparation of NaOH and sterilization in autoclave; *
4. Assembly of fermenter under laminar flow hood;

5. Addition of water in the reactor, heat to 50°C, add amylase, rice flour and glucose;
6. Heat to 85°C and hold for 20 min to allow hydrolysis of starch;
7. Sterilize in autoclave;
8. The fermenter is linked to the water bath and peristaltic pump;
9. Adjustment of temperature and pH;
10. Inoculation;
11. Manual sampling every two hours;
12. After 24 h end of fermentation;
13. Analysis;
14. Stabilization of fermented matrix (90°C for 1 min);
15. Freeze-drying;(A.4)
16. Analysis of dried samples;
17. In vitro analysis;

* only for pH controlled fermentations

3.3.4 Rice flour supernatant's fermentations' protocol

The protocol used is the same as the one used for rice flour fermentations until step 7. Once the sterilization takes place the following steps are:

1. Cool suspension inside fermenter to room temperature;
2. Centrifuge suspension to separate solid and liquid (two centrifugal speeds-1000 rpm and 2000 rpm- for 15 min);
3. Recover supernatant;
4. Sterilize in autoclave;
5. Link the fermenter to the water bath and peristaltic pump;
6. Adjustment of temperature;
7. Inoculation;
8. Manual sampling every two hours, with total duration of the process for 24h.

To stabilize the suspension and therefore reduce the microbial charge to less than 10^2 CFU/mL, the suspension is heated to 90°C for 1 minute inside the reactor.

Once the suspension has cooled to room temperature, samples of 10 mL each, are frozen at -26°C for at least 24h before dehydration. Dehydration for all substrates through freeze-drying (A.4) takes up 24 h obtaining a dry powder at the end of the process.

3.4 Analytical methods

To verify the outcome of each fermentation, several analyses were performed. These include microorganisms' growth detection, organic

acids measurement, active compound detection and sugar consumption.

In the following paragraph each method used at lab scale and the treatment of the samples whenever needed will be explained;

3.4.1 Spread plate method

To detect the growth of *L. paracasei* and eventual presence of contaminations the direct plate count method, more specifically the spread plate method (A.1) was used

A sample of the fermented matrices of 0.1 mL was diluted through serial dilution (A.5) then sewed onto agar dishes. The plates used contained three main kinds of medium: MRS (Sigma-Aldrich) agar to control the growth of *L. paracasei CBAL74*, Gelisate (Biolife) and MacConkey (Sigma-Aldrich) agars to detect the presence of contaminants.

3.4.2 Analysis of primary and secondary metabolites

During lactic acid fermentations, the main acid produced is lactic acid. Its concentration was measured throughout the fermentations using HPLC. Furthermore, the samples were also analysed to detect the presence of different organic acids such as butyric, acetic, and propionic acids using GC.

HPLC

To analyse and quantify lactic acid (main metabolite of the fermentation process) the following procedure was followed:

- Weigh 2 g of sample;
- Add 1 mL of 0.1 M of potassium ferricyanide (III)- $K_3Fe(CN)_6$ (0.1M), 1 mL of zinc sulfate heptahydrate- $ZnSO_4(H_2O)_7$ (0.2M) and 2 mL of sodium hydroxide- NaOH (0.1M);
- Add H_2O until total volume reaches 50 mL;
- Vortex;
- Leave overnight at 4°C;
- Next day: centrifuge at 3000 rpm for 10 min;
- Filter the supernatant using paper filters (Whatman 90 mm);
- Collect the supernatant;

The mobile phase used was a solution of ammonium dihydrogen phosphate (0.09 M) with a pH of 2.7.

The column used was Agilent Zorbax C18 (4.6 mm X 150 mm) with pore size of 80 A. The flow rate was set at 0.8 mL/min with detection of the substance at 218 nm.

GC

Gas chromatography is a type of chromatography used in analytical chemistry for separating and analysing compounds that can be vaporized without decomposition. This method was used to detect the presence of acetic, butyric and propionic acids that are produced by competitive bacteria and indicate the presence of contaminations.
Samples were treated the same way as for lactic acid before injection into the column.

The instrument used was GC Agilent technologies 8690, with capillary Q column (25 mm X0.23 mm) and the mobile phase used was Helium, with flow rate 200 mL/min and the internal standard was esanoic acid at 5 g/L.

3.4.3 Analysis of sugar consumption

HPLC was used to measure the quantity of the various sugar content in the samples. Samples were treated before injection into the HPLC following the same procedure for lactic acid analysis.

The instrument used was Agilent technologies 1260. The column used was phenomenex Rezex RHM monosaccharide H+(LC column 300 X 7.8 mm). The mobile phase was water with 3.5 mM of sulphuric acid.

3.4.4 Analysis of postbiotic/ active metabolite

The identification of the active metabolite, its nature and its sequence was done using the Maldi-TOF method (A.2). Subsequently, RP-HPLC (A.6) was used as a method for routine analysis.

3.4.4.1 Maldi-Tof

Pretreatment of the sample

Samples were first pretreated in various ways to identify the best method to concentrate and purify the peptide. These methods were tested in combination as following:

- Molecular weight/ molecular nature: in this case, samples were filtered using Amicon® ultra 0.5 mL filters which allowed the separation of molecules based on their MW, then the pellet (containing molecules with low MW) was treated with various solvents (methanol, chloroform).
- Molecular Nature/ hydrophobic interactions: in this case, samples were treated with various solvents (TFA, methanol...)

then the peptide was extracted using solid phase extractions (C18 columns).

After initial treatment, proteins in the samples were separated by gel electrophoresis (A 7). After which, the gel was stained using the Coomassie Blue 250 for initial analyses followed by staining by silver stain. Gels were then scanned and analyzed.

The staining was then removed and samples were prepared for quantification.

Analysis

To perform such analysis, a standard synthetic peptide was ordered in two states: normal (1120 Da) and labeled on the phenylalanine (1130 Da). The protocol used is reported below:

1. Cut bands from gel, clean with water and incubate at 37°C for 20 minutes;
2. Remove water and add 200 µL of de-stain solution;
3. Wash with 200 µL of washing solution and incubate for 30 min at 37°C;
4. Wash with water;
5. Incubate with acetonitrile 100 % for 2 min;
6. Remove ACN;
7. Add 100 µL of dithiotheritol (DTT) 10 M, and incubate at 56°C for 45 min;
8. Remove DTT and add 100 µL of indole-3-acetic acid (IAA);
9. Remove IAA solution and add 200 µL of washing solution for 20 min at 37°C; repeat twice;
10. Add 100 µL of ACN for 2 min, then remove;
11. Add 35 µL of trypsin in ice bath for 20 min, then overnight at 37°C;
12. The next day add trifluoroacetic acid (10%);
13. Prepare matrix for CHCA/thin layer:
    a. Solution 1: 10 mg HCCA=50:50 ACN: EtOH
    b. Solution 2: 3.5 mg HCCA= 50:50 ACN: H_2O
14. Spot sample on matrix using ziptip pipette tips;
15. Spot standard mix solution at the center of each square;
16. Run analysis.

3.4.4.2 RP-HPLC

Each fermented substrate was treated differently, as follows:

- *Pre-treatment of milk fermented samples*

1.Mix sample with TFA (0.2%) in a 1:1 ratio;

2.Centrifuge at 5000 rpm for 20 min.;
3.Collect supernatant;
4.Filter supernatant using Amicon® Ultra filters (3K) and centrifuge at 14.000 x g for 30 min;
5.Collect the filtrate;
6. HPLC analysis;

- *Pre-treatment of rice flour fermented samples*

1.Centrifuge sample at 5000 rpm for 20 min;
2.Collect supernatant and filter using Whatman® filters (D:9 cm, pore size 11 µm);
3. HPLC analysis;

The HPLC used was an Agilent 1100 binary pump, with a C18 column (Synergi™ 4µm Fusion –RP80A°, 250 x 4.6 mm). The detection was at 215 nm. Two buffers were used for the mobile phase:

- Buffer A: 0.05%TFA+2% CH_3CN
- Buffer B: 0.05%TFA+90% CH_3CN

With a gradient of 10-20% of B in 22 min. These analyses were performed at 30°C.

In order to perform such analysis on dried powder of fermented milk or rice flour, the powder was suspended in HPLC pure water maintaining the same initial proportions of fermentation recipes (10% for milk and 15% for rice flour) then the procedure was the same as the pretreatment mentioned above.

3.4.5 Supernatant fractions for in vitro trials

LP CBA L74 supernatants were fractioned using Amicon Ultra-15 10 K Centrifugal Filter Devices (Millipore, Milano, Italy). 15 mL of supernatant were added to the Amicon Ultra filter device and centrifugation (4000 rpm for 40 min) was performed to separate the fractions. Both <10 and > 10 kDa were used.

3.4.6 Culture substrates

Fermented rice flour samples, obtained after fermentation performed with and without pH control, were freeze-dried after inactivation of bacteria by heat treatment (85°C for 20 s). The control powder consisted of rice flour processed with a similar procedure but without the inoculation step.
The freeze dried samples (fermented and control) were diluted in DMEM (GIBCO, San Giuliano Milanese, Italy) and stirred at 37°C for about 15 min. The solution obtained was centrifuged at 3000 rpm for 10 min at RT to recover supernatant necessary for cell contact.

3.4.7 Fluorescence assay

Cells, grown in sterile glass coverslips, were transferred into a 24-well plate and treated with the supernatant for 30 min. After this time, two different cases were studied.

- Case 1: Treatment of Caco-2 cells with lissaminated P31-43 in 5% CO_2 atmosphere at 37°C, for 30 min.
- Case 2: Identical to the first trial, but performed after a robust washing with PBS, in order to verify a possible memory effect on the cells due to the supernatant treatment.

Peptides were used in the following concentrations: P31-43-lissamine at 20 µg/mL; unlabelled P31-43 peptides were used at 80 µg/mL. After washing in PBS, the coverslips were briefly fixed (5 min) with paraformaldehyde 3% (Sigma-Aldrich, Milan, Italy) at room temperature. The coverslips, after mounting on glass slides, were observed by confocal microscope (LSM 510 Zeiss, Milan, Italy), and images were analysed with AIS Zeiss software to evaluate the intensity of fluorescence (FI) of the microscopic field under consideration [70]–[73]. Magnification of the micrographs was the same for all the figures shown (63 objective).

Both gliadin peptide P31-43 and P31-43 linked to fluorochrome Lissamine (wavelength 575/595) were synthetized by InBios, Napoli, Italy. The peptides, obtained by Ultrasart-D20 (Sartorius AG, Goettingen, Germany) filtration, were 95% pure, as analysed by MALDI-tof (A.2). P31-43 sequence was LGQQQPFPPQQPY

3.5 Fermentation kinetics

3.5.1 Fermentation parameters

Reported in this paragraph are the various fermentation parameters assessed for milk and rice flour fermentations at laboratory scale. These include doubling time (td), generation number (n), constant of growth rate (K), biomass yield ($Y^{X}/_{S}$) and lactic acid yield ($Y^{P}/_{S}$).

The time needed for the microorganism to double in number during the exponential phase is known as the doubling time and is calculated using Eq (1)

$$td = \frac{t\exp}{n} \quad (1)$$

where t_{exp} is the duration of the exponential phase, n the number of generations produced. n is calculated using Eq (2)

$$Nf = N0 * 2^{n} \quad (2)$$

where N_f and N_0 are the number of cells at the end and beginning of the exponential phase t_{exp} respectively.

The constant of growth rate (K) is calculated by using Eq (3).

$$K = \frac{n}{t\ exp} = \frac{1}{td} \quad (3)$$

The amount of biomass produced to the amount of substrate consumed during the growth phase $Y^{x}/_{S}$ is calculated using the following Eq (4)

$$Y^{x}/_{S} = \frac{\Delta X}{\Delta S} = \frac{X-X0}{S-S0} \quad (4)$$

where X_0 is the initial concentration of biomass in g/L, X the final concentration of the biomass at time t (g/L), S_0 the initial concentration of substrate g/L and S the concentration of substrate at time t (g/L).

The amount of lactic acid produced to the amount of substrate consumed $Y^{P}/_{S}$ is calculated using the following Eq (5)

$$Y^{P}/_{S} = \frac{\Delta P}{\Delta S} = \frac{P-P0}{S-S0} \quad (5)$$

where P_0 is the initial concentration of lactic acid (g/L), P the concentration of lactic acid at time t (g/L), S_0 the initial concentration of substrate (g/L) and S the concentration of lactic acid at time t (g/L).

3.5.2 Mathematical modelling

The batch fermentation process can be modelled by the mass balances on biomass [X], lactic acid produced [P], and on substrate [S]. Kinetic unsegregated and unstructured models are proposed to describe the growth biomass, product formation and substrate consumption rates.

3.5.2.1 Cell growth kinetics

The cell growth rate can be expressed by a first order kinetic model respect to the concentration of cells according to Eq (6)

$$rx = \frac{dX}{dt} = \mu\, X \quad (6)$$

where μ is the specific growth rate (1/h). assuming μ constant under the growth conditions investigated, the integration of Eq (6) setting the initial condition $X=X_0$ at $t=t_0$ – yields Eq (7)

$$\mu = \frac{ln\,(\frac{x}{x0})}{t-t0} \quad (7)$$

where t_0 is the initial time of exponential phase and X_0 the corresponding biomass concentration.

The unsegregated-unstructured kinetic model used to express the specific growth rate as a function of the limiting substrate is the Monod's equation Eq (8)

$$\mu = \mu\max\frac{S}{S+Ks} \quad (8)$$

where μmax, S, and Ks are the maximum specific growth rate (1/h), the limiting substrate concentration (g/L) and the semi saturation constant (g/L) respectively. Lactic acid inhibition is not considered due to pH control.

In order to calculate the Monod kinetic coefficients μmax, S, and Ks two linearization methods are proposed: 1. Lineweaver-Burk linearization method and 2. Hanes' linearization method as following

$$\frac{1}{\mu} = \frac{Ks}{\mu\max} * \frac{1}{S} + \frac{1}{\mu\max} \quad (9)$$

$$\frac{S}{\mu} = \frac{Ks}{\mu\max} + \frac{S}{\mu\max} \quad (10)$$

In the case of Lineweaver- Burk's method, a plot of 1/μ versus 1/S provides a linear line with a slope of Ks/μ and a y-axis intercept of 1/ μmax.

Hanes linearization should provide a greater precision of kinetic coefficients' estimation than Lineweaver-Burk's method in case of low values of μ. In this case S/ μ is plotted versus S, a linear regression is applied and the slope and the y-axis intercept values are used to calculate Ks and μmax.

3.5.2.2 Biomass and lactic acid productivities

The productivities of the fermentation process referred to the biomass (Wx [mg/L*h]) and lactic acid (Wp [mg/L*h]) are evaluated according to Eq (11) and Eq (12).

$$WX = \frac{\Delta X * V}{tdead + t} \quad (11)$$

$$WP = \frac{\Delta P * V}{\mathrm{tdead+t}} \quad (12)$$

where V (L) is the total volume of suspension, ΔX (L/mg) and ΔP (mg/L) the amounts of biomass and lactic acid produced during process respectively, t dead (h) the dead time of the fermentation process and t (h) the time of process.

PILOT SCALE

3.6 Materials

3.6.1 Fermentation feedstocks

The feedstocks used to perform the fermentations at pilot scale were powder milk and rice flour.

Powder milk: the nutritional values of milk powder in general per 1 cup (68 g) is reported in the following table (13)

Table 13: Nutritional value of milk powder for 68 g (1 cup)

Nutritional value of milk powder for 68 g	
Total fat	0.5 g
- Of which saturated fat	0.3 g
- Of which polyunsaturated fat	0 g
- Of which monounsaturated fat	0.1 g
- Cholesterol	12 mg
Total carbohydrates	35 g
Dietary fiber	0 g
Sugars	35 g
Protein	24
Sodium	373 mg
Potassium	1159 mg

Rice flour: the rice flour used for these fermentations is the same used at laboratory scale. (2.1.2).

3.6.2 Fermentation recipes

The recipes used to perform the fermentations are reported below in table (14) and (15) for milk and rice flour fermentations respectively:

Table 14: Recipe for milk fermentations:

Ingredient	percentage
Milk (powder)	10.5%
Glucose	2%
Water	87.5%

Table 15: Recipe for rice flour fermentations:

Ingredient	Percentage
Rice flour	15%
Glucose	1%
Amylase	0.015%
Water	84%

3.7 Apparatus

The pilot plant used to perform the fermentation trials is currently present in the Heinz Innovation Centre located in Nijmegen, The Netherlands. Figures (9) (10)

The plant consists of 6 main unit operations as follows: Powders/ slurry preparation mixing tank; heating system; buffering; cooling; fermenter and spray dryer.

The mixing tank (a) has a maximum volume of 500 L and it is directly connected with line water supply (the remaining fermentation ingredients are measured outside and then added manually). The product is sent from the mixing tank to the buffering tank (c) by a screw pump with a flow rate of 200 L/h passing through tubes (b) in which the first direct steam injection takes place. The plant is provided with a saturated steam of 4 bar and the quantity added is controlled by a PID (A.8) feedback control. The temperature is controlled by a temperature probe located at the end of the steam injection; the integrated PLC reads the temperature and with a feedback control adjusts the amount of steam needed to heat up the product regulating the steam valve. The buffering tank (c) has a maximum capacity of 300 L and it has an external jacket supplied with water to control the temperature of the product inside, it also has an agitation system. The buffering tank is connected with the fermenter through tubes (d) where the second steam injection and the cooling phase (e) take place. The pump used to send the product from the buffering tank to the fermenter was set to 250 L/h flow rate. The cooling takes place in a tube in tube heat exchanger in which the exterior tube is provided by water at 20°C. The fermenter (f) has a maximum capacity of 300L with an impeller with

customized inclined blade turbine connected to a motor that allows the control of the mixing speed. It has an external jacket that controls the temperature inside. The fermenter has 4 input points connected to:

- probe (Mettler pH-electrode InPro3100/120) that measures the pH and temperature simultaneously with the progress of the process;
- 2 peristaltic pumps to supply inoculum and sodium hydroxide;
- air compressor to control the pressure inside the reactor.

The fermenter has a valve in the lower part, connected to low pressure steam that allows sterile sampling throughout the process.
The spray dryer (g) (A.9) is connected to the fermenter through an external pump. It is a vertical axis spray dryer with a co-flow system and a pressurized nozzle. At the bottom of the cyclone the product is collected in a bucket (h). The throughput of the spray dryer is 0,45 kg powder/hour.

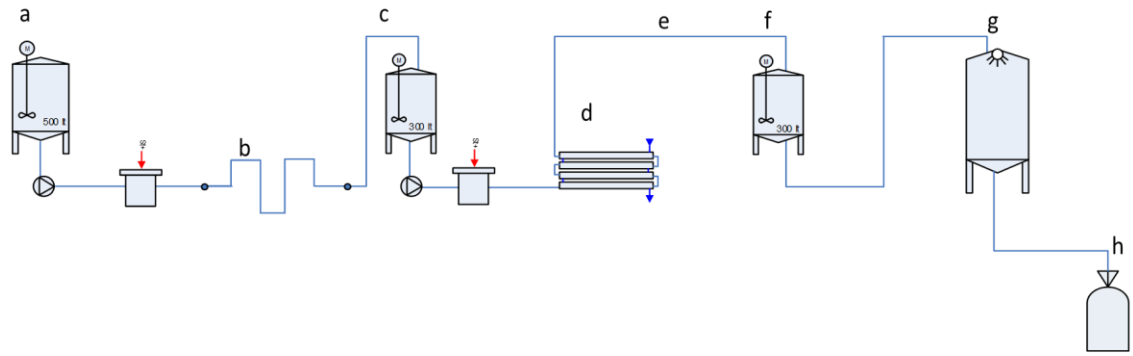


Figure 9: Pilot plant process flow diagram: the plant contains six main operation units.

Figure 10: Pilot plant in the Kraft Heinz innovation centre (57), Nijmegen, The Netherlands

3.8 Experimental methods

3.8.1 Strain's propagation:

The preparation of the inoculum at pilot scale is a two-step process. The first step consists in preparing the pre-inoculum from a working cell bank (A.10), adding a single glass bead under laminar flow hood and transfer to 15 mL falcon tube containing 10 mL of BAF. Incubate at 37°C for 24 h. whereas, the second step consists in the transfer of the pre-inoculum into a Schott type glass bottle (5L) containing 2 L of BAF (previously sterilized). Incubate at 37°C for 24h.

3.8.2 Protocol:

Pilot scale tests were performed for two substrates: milk and rice flour. The process and recipes developed at lab scale were scaled up and adapted to the pilot scale. The percentages of the ingredients in the fermenter were unvaried.

Each fermentation test performed at pilot scale had a duration of 5 days. In the following paragraph are the steps during each day:

Day 1:

- First step of inoculum preparation: reactivation of *L. paracasei* in 9 mL BAF and incubation at 37°C for 24h under laminar flow hood;

Day 2:

- Second step of inoculum preparation: transfer inoculum into 2L of BAF and incubation at 37°C for 24h under laminar flow hood;
- Preparation of NaOH (30% w/v) and sterilization;
- Sterilization of valves;

Day 3:

- Attachment of autoclaved valves to inoculum Pyrex container and NaOH container;
- Drainage and filling of mechanical seals;
- Calibration of pH meter;
- Air filters control;
- Sterilization of SPX (UHT) line;
- Deaeration of fermenter;
- Connection and steam flushing of all valves;
- Sterilization of fermenter (121°C/30 min);
- Measurement of all ingredients;
- Addition of ingredients into mixing tank;
- pH Control;
- Transfer of product to buffering tank through tubes where the first direct steam injection (85-90°C) takes place;

- Holding of the product in buffering tank (for 20 min) *;
- Transfer of product to fermenter through tubes where the second direct steam injection (136°C) followed by cooling to (37°C) take place;
- Inoculation of *L. paracasei* into the fermenter;
- Start the fermentation process;
- Sampling every two hours to monitor the process;

Day 4:

- End of fermentation process;
- Pasteurization of the product inside the fermenter at 80°C for 30 s;
- Cooling (37°C);
- Spray drying of the product;
- Collection of final product;

Day 5:

- Cleaning of all pilot plant.

*This step is only done for rice flour fermentations

3.9 Analytical methods

3.9.1 Pour plate method

To detect the growth of *L. paracasei* and eventual presence of contaminations double pour plate method (A.1) was used.
A sample of the fermented matrices of 0.1 mL was diluted using serial dilution (A.5) then sewed onto agar dishes. The plates used contained three main kinds of medium: MRS agar (Sigma-Aldrich) to control the growth of *L. paracasei CBA L74*, Gelisate (Biolife) and MacConkey agars (Sigma-Aldrich) to detect the presence of contaminants.

3.9.2 Analysis of primary metabolite

To quantify the amount of lactic acid produced at pilot scale, YSI 2900 Biochemistry analyser was used (A.11). Samples processed by the YSI were prepared as following:

- Dilute sample with sterile demi-water 1:1;
- Vortex;
- Measure.

3.9.3 Shelf life analysis

To detect the amount of time the dried product (whether fermented rice flour or milk) may be stored without alteration of the amount of lactic acid and active metabolite, shelf life analysis was performed. To perform such analysis, samples from each fermented dried product

were stored at three different temperatures: 4°C, 20°C and 37°C. Analysis were then performed at a monthly basis following the protocols previously described (3.4.2) and (3.4.4) for lactic acid and active metabolite respectively.

3.10 Statistical analysis

Statistical analysis and graphics were obtained from GraphPad Prism (San Diego, CA). Mean and standard deviation of the experiments were calculated; their significance evaluated by Student's *t*-test accepting as significant only results that showed values of $p < 0.05$.

Chapter IV

Results and Discussion

The main results are reported in this chapter. The first part regards the results reported at lab scale, whereas the second part reports the pilot plant scale results, the third and final part is a comparison between the two scales.

4.1 Laboratory scale results

4.1.1 Milk fermentation

Milk fermentation was performed following protocol, fermentation system and recipe described in section (3.3.2) (3.2.1) (3.1.3) respectively. As mentioned in the introduction, milk fermentations were carried with and without pH control, to understand if the pH could have effects on bacterial growth, on the metabolites' production (primary and secondary) and on the active metabolites. For the protocol assessed as the best, carbon (sugar) consumption, stabilized products' analysis, fermentation parameters and kinetics were also studied. Taking into account the volume of the fermenter (1.5 L), a limited number of samples was withdrawn during each fermentation test to ensure no influence on the hydrodynamics and pH measurements. As a consequence, to assess a full fermentation curve a minimum of 4 fermentations were necessary. All tests lasted 24 h.

4.1.1.1 Fermentations without pH control

These fermentations started with initial pH value (t_0) of 6.6 ± 0.03 and dropped to 5.44 ± 0.08 at t_{24}. Figure (11) reports the pH vs fermentation time measured.

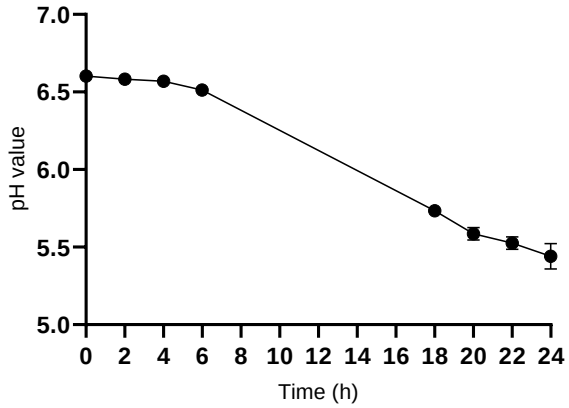


Figure 11: pH vs time measured during milk fermentation carried without pH control.

In figure (12) the growth curve is shown, the fermentation had a lag phase of 2 h, an exponential phase that lasted for 18 h, and the

stationary phase started after 20 h. Fermentations started (t_0) with an initial bacterial charge of 1.09E+06 ± 4.45E+05 CFU/mL and a final (t_{24}) bacterial charge of 2.8E+08 ± 4.9E+07 CFU/mL reaching the maximum bacterial charge at t_{20} with a charge of 4.50E+08 ± 1.35E+08 CFU/mL.

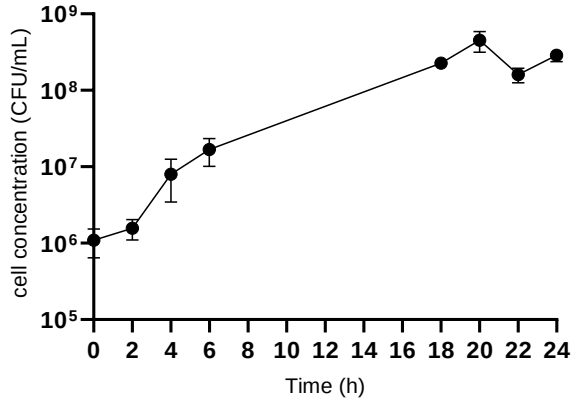


Figure 12: Cell concentration vs. time measured during milk fermentations carried without pH control.

The primary metabolite produced during the fermentations was lactic acid. Its concentration was measured as described before (2.4.2). The production of lactic acid started after 4 h of fermentation (t_4) with a concentration of 0.32 ± 0.04 g/L and increased to 3.5 ± 0.05g/L at t_{24}. (Figure 13). Butyric, acetic and propionic acids were not found. (Figure 13)

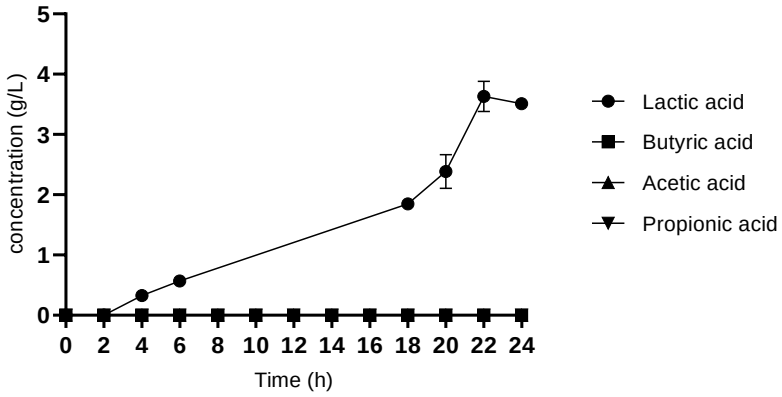


Figure 13: Organic acids' concentration vs fermentation time measured during milk fermentations carried without pH control.

4.1.1.2 Fermentations with pH control

Fermentation tests were carried out with pH at 6.76 ± 0.05 (pH of milk) and set at 6.2 after 10 h of fermentation to maintain pH at the optimal levels for the growth. The control was provided by feeding a NaOH 0.2 M solution by means of a peristaltic pump.

Figure (14) shows pH vs time profile measured during the fermentations.

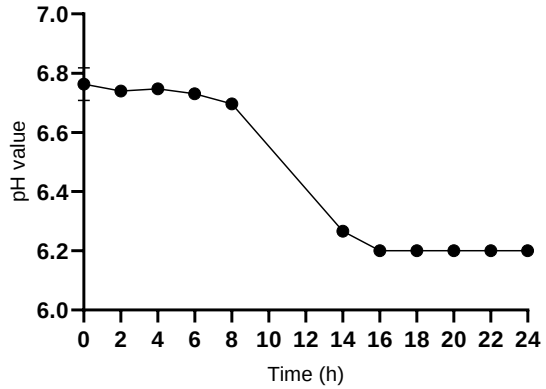


Figure 14: pH vs fermentation time measured during milk fermentations under pH control.

The figure (15) reports the cell concentration vs. time measured during the fermentation time. A lag phase of 4h, an exponential phase of 10h and a stationary phase that started after 14h of fermentation are shown. The mean value of initial bacterial charge was 2.40E+06 ± 2E+05 CFU/mL reaching a charge of 1.6E+08 ± 3E+07 CFU/mL after 24h of fermentations. The highest bacterial charge was reached at 14h of fermentation with a value of 1.66E+08 ± 3.5E+07 CFU/mL.

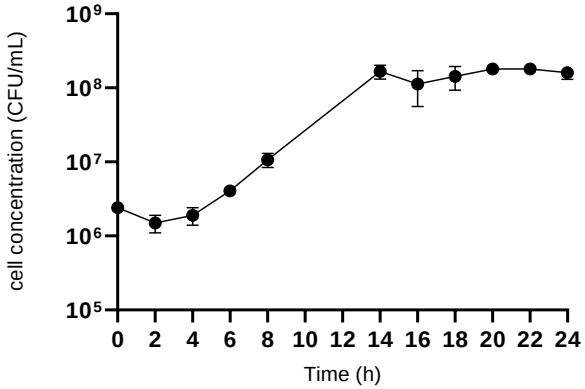


Figure 15: Cell concentration vs. time registered during milk fermentations under pH control.

Lactic acid's concentration was 0.04 ± 0.006 g/L measured after 14h of fermentation, increasing to a value of 1.3 ± 0.0 g/L after 24h. The concentrations of butyric, acetic and propionic acids were also measured with no traces found. (Figure 16)

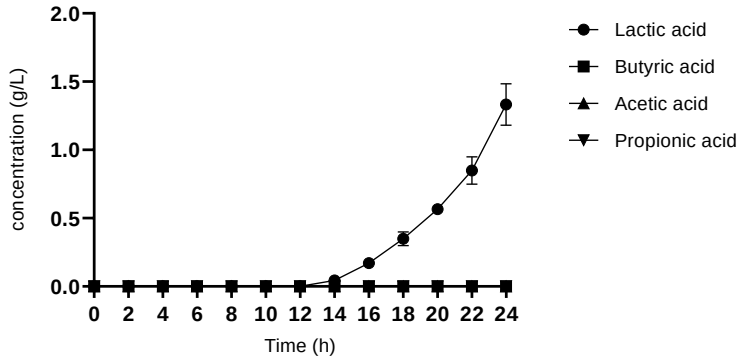


Figure 16: Organic acids' concentration vs time measured during milk fermentations under pH control.

On the basis of the results just described, the best protocol was with pH control, thus it was chosen as the standard fermentation protocol.

Characterization of the fermentation under pH control

Sugar concentrations were measured using HPLC. Three sugars were analyzed in milk fermented samples: lactose, glucose and sucrose. Figure (17) reports the consumption of these sugars vs. fermentation time. Around 6 g/L of glucose, 0.8 g/L of sucrose and 0 g/L of lactose were consumed throughout the 24h of fermentations.
It is then possible to assume based on these results that glucose is the metabolized substrate because of the lower consumption of other sugars.

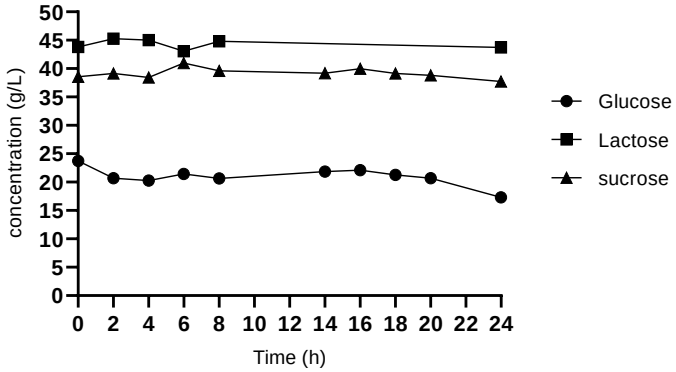


Figure 17: Sugar concentration vs fermentation time measured during milk fermentations under pH control.

Figure (18) shows the accumulation curve of the active metabolite in time. During milk fermentations the active compound was detected after 16 h of fermentation with a concentration of 1.29 ± 0.1 mg/L and increased up to 6.9 ± 0.5 mg/L at 24h of fermentation.

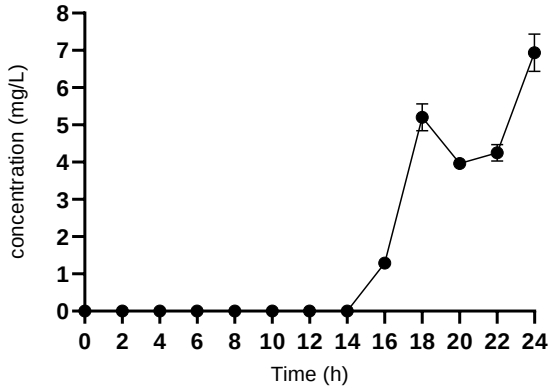


Figure 18: Active metabolite's concentration vs fermentation time measured during milk fermentation under pH control.

Figures (19) and (20) report the biomass yield ($Y_{X/S}$) vs. fermentation time and product (lactic acid) yield ($Y_{P/S}$) vs fermentation time respectively.

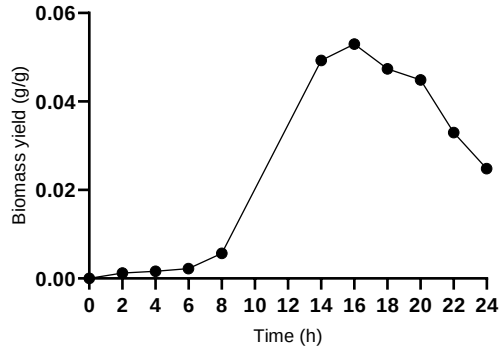


Figure 19: Biomass yield (Y X/S) vs fermentation time for milk fermentations carried under pH control.

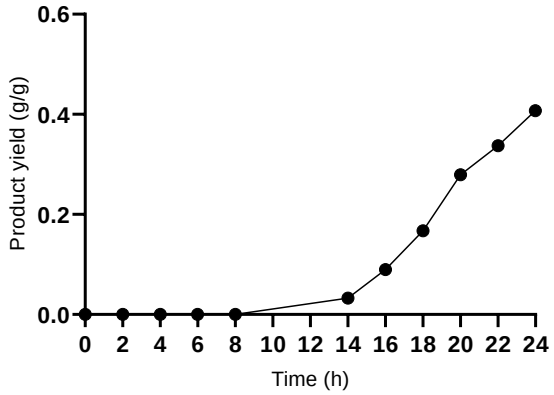


Figure 20: Product yield (Y P/S) vs fermentation time for milk fermentations carried out under pH control.

$Y_{X/S}$ (biomass yield) has a maximum after 16h of fermentation with a value of 0.05 g/g (5%), whereas $Y_{P/S}$ (lactic acid yield) has a maximum value after 24h of fermentation with a value of 0.039 g/g (3.9%).

Doubling time (td), constant growth rate (K), generation number, final biomass yield and the product yields are reported in table (16). Both the biomass yield and product yield are evaluated respect to total carbon (glucose) substrate utilization after 24h of fermentation.

Table 16: Fermentation parameters: doubling time (td), constant growth rate (K), generation number (n), final biomass yield ($Y_{X/S}$) and Lactic acid yield ($Y_{P/S}$)

td (h)	K (h^{-1})	n	$Y_{X/S\ (t=24h)}$ (g/g)	$Y_{P/S(t=24h)}$ (g/g)
2.54	0.39	7.09	0.02	0.23

Monod's model was linearized according to Lineweaver-Burk;

$$\frac{1}{\mu} = \frac{Ks}{\mu max} * \frac{1}{S} + \frac{1}{\mu max}$$

Lineweaver- Burk's linearization gave a R^2 0.97 and the values for Ks and μmax were obtained by linear regression and reported in the table (17).

Table 17: Values of Ks and μmax obtained for Monod's model

μmax (h^{-1})	Ks (g/L)
0.3238	0.1647

These results show that the quantity of glucose present highly satisfies the microorganism's need because S >> Ks.

Biomass and lactic acid productivities (W_X and W_P) have been calculated according to Eq (11) and Eq (12), where the dead time has been set to zero. W_X increased with time and approached the maximum (0.01 g/h) after 20h of fermentation. Figure (21)

Lactic acid productivity (W_p) referred to substrate consumption during fermentation process is showed in figure (22). The productivity increased with the time and the maximum-measured at the end of the fermentation test- was 0.039 g/h.

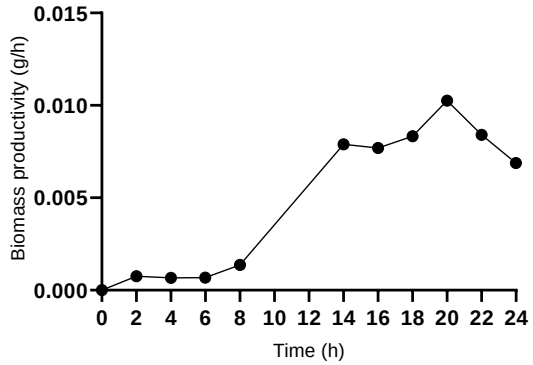


Figure 21: Biomass productivity vs fermentation time for milk fermentations carried out under pH control.

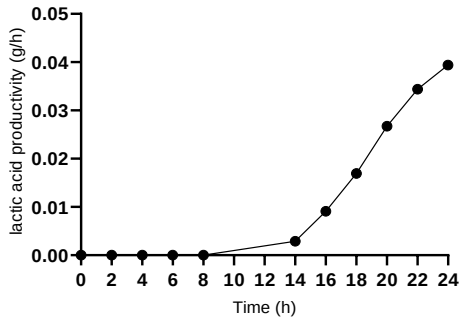


Figure 22: Lactic acid productivity vs fermentation time for milk fermentations carried out under pH control.

4.1.2 Rice flour fermentations

Rice flour fermentations were performed following the protocol, system, and recipe described in sections (3.3.3) (3.2.1) (3.1.3) respectively. For this substrate, fermentation tests were performed with and without pH control as well. Furthermore, fermentations were also performed using the supernatant obtained from the rice flour and water suspension.

For both protocols bacterial growth and organic acids production was studied. Also in this case, the protocol considered to be the best was further analyzed for active metabolites, carbon consumption, stabilized products, fermentation parameters and kinetics.

4.1.2.1 Rice flour fermentations without pH control

These fermentations started with initial pH values (t_0) of 5.8 ± 0.02 and dropped to 3.5 ± 0.0 after 24h of fermentation. Figure (23) reports the pH vs. fermentation time.

Figure (24) shows the growth curve with a lag phase of 4 h, and an exponential phase that lasted for 10 h, the stationary phase started after 14 h of fermentation. The mean bacterial charge at the beginning of the fermentation was 2.60E+06 ± 2.82E+05 CFU/mL increasing to 5.2E+08 ± 9.1E+07 CFU/mL which also corresponded to the highest charge registered.

The primary metabolite produced during the fermentations was lactic acid. Its concentration was measured as described in section (2.4.2). The production of lactic acid started after 14h of fermentation (t_{14}) with a concentration of 0.62 ± 0.01g/L and increased to 1.9 ± 0.07 g/L. Figure (25).

Butyric, acetic and propionic acids were not found. (Figure 25).

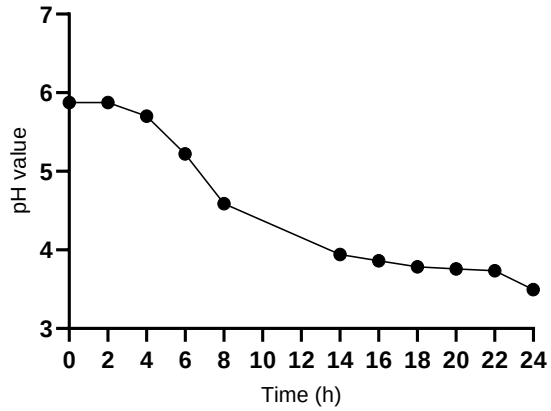


Figure 23: pH vs fermentation time measured during rice flour fermentations without pH control.

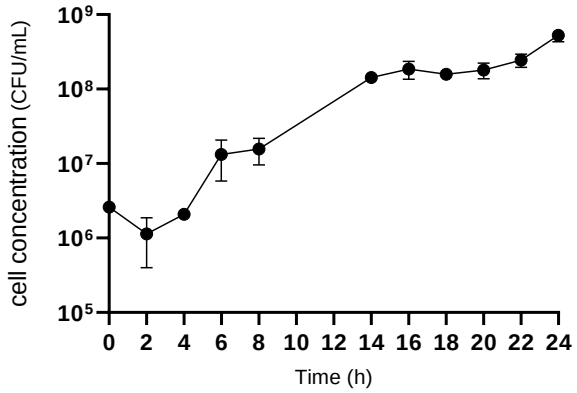


Figure 24: Cell concentration vs fermentation time measured during fermentation without pH control.

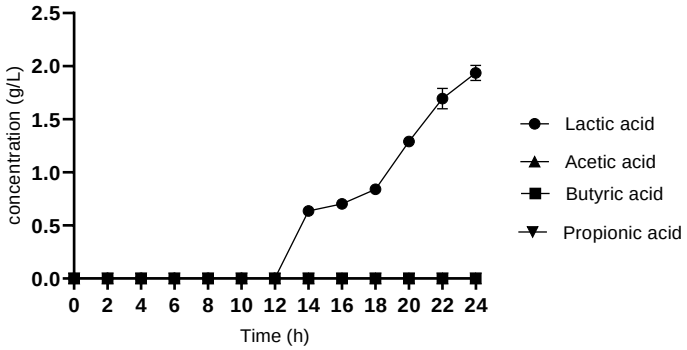


Figure 25: Organic acids' concentration vs fermentation time measured during rice flour fermentation without pH control.

4.1.2.2 Rice flour fermentations with pH control

Fermentation tests were performed setting and maintaining pH values to 6.2. The suspension of rice flour and water has an initial pH of 5.8. Trials done controlling the pH by feeding NaOH 0.2 M solution by means of a peristaltic pump after inoculation of bacteria resulted in the decrease of bacterial charge for the first 4h followed by a steady growth. To avoid such initial decrease in bacterial charge, pH was adjusted and stabilized for at least 20 min before inoculation. Tests were then performed at pH 6.2 for the 24h.

Figure (26) reports the cell concentration vs. fermentation time measured during the fermentation tests. A lag phase of 4h, an exponential phase of 14h and a stationary phase that started after 18h of fermentation are shown. The mean value of initial bacterial charge was 2.5E+06 ± 8.5E+05 CFU/mL reaching a charge of 2.26E+08 ± 1.14E+08 CFU/mL at 24h, with a maximum growth of 5.3E+08 ± 1.47E+08 CFU/mL registered after 18h.

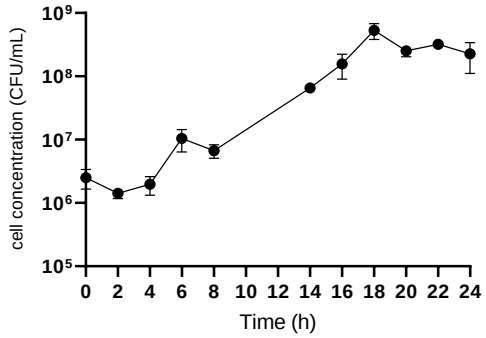


Figure 26: Cell concentration vs. fermentation time measured during rice flour fermentation with pH control.

Lactic acid's production was measured around 14h of fermentation with a value of 1.13 ± 0.19 g/L. The final amount at 24h was 4.4 ± 0.21 g/L. Butyric, acetic and propionic acids were also measured with no traces found. Figure (27) reports the concentration of the organic acids vs. fermentation time.

On the basis of the results obtained, the best protocol in terms of bacterial growth and lactic acid production is pH controlled rice flour fermentation.

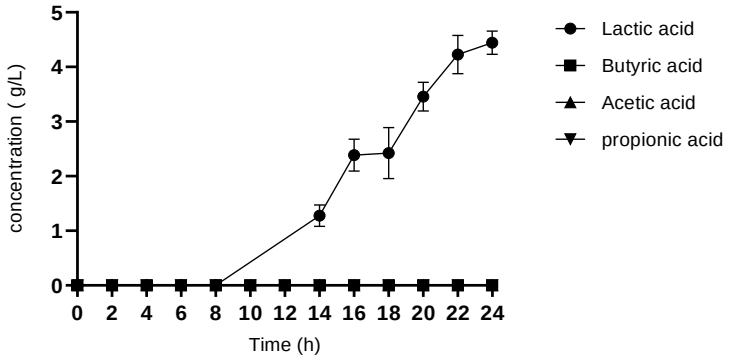


Figure 27: Organic acids'concnetration vs. fermentation time measured during rice flour fermentation with pH control.

Charaterization of the fermentation under pH control

Carbon immediate source in rice flour was free sugar that was measured throughout the process. The broth contained a minimal quantity of sucrose (initial quantity of 4.88 g/L), glucose (14.3g/L) and a minor concentration of fructose (3.24 g/L). During the fermentation fructose was completely consumed, mainly during the first two hours of fermentation, a minimal amount of sucrose was consumed, while almost 10 g/L (76.75%) of glucose was consumed. (figure 28)

It's possible to consider the glucose as the main metabolized substrate, due to the higher quantity consumed when compared to other sugars.

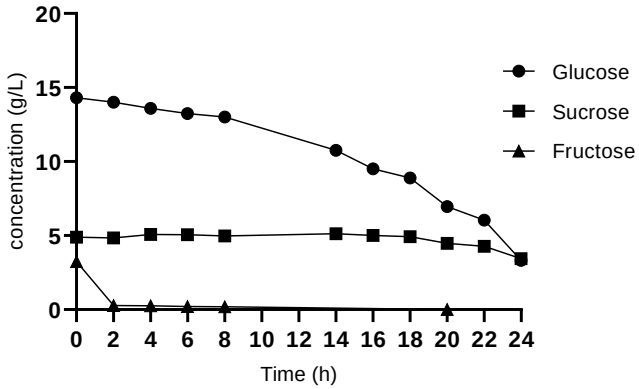


Figure 28: Sugar concentration vs fermentation time measured during rice flour fermentation with pH control.

Figure (29) reports the accumulation trend of the active metabolites vs fermentation time. Production of the active metabolite during

fermentation was detected around 14h of fermentation with a concentration of 13.7 ± 1.9 mg/L. The quantity increased reaching the amount of 17.2 ± 0.35 mg/L. During the initial hours of fermentation, the concentration of such metabolites was not detectable.

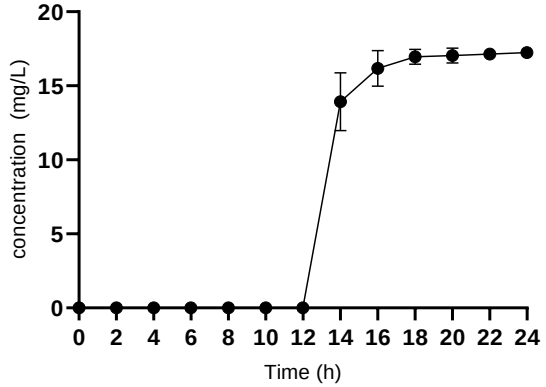


Figure 29:Active metabolite concentration vs fermentation time measured during rice flour fermentation with pH control.

Biomass yield ($Y_{X/S}$) and the lactic acid production yield ($Y_{P/S}$) during the fermentation are shown in figures (30) and (31) respectively. $Y_{X/S}$ reaches a maximum value of 0.1 g/g after 18h of fermentation, while $Y_{P/S}$ starts a stationary regime with an almost constant value of 0.5 g/g. In table (18) are reported the doubling time (td), generation number (n), the constant of growth rate (K) and the final biomass and product Yields. $Y_{X/S}$ and $Y_{P/S}$ were evaluated respect to the total carbon substrate utilization (glucose) after 24h of fermentation.

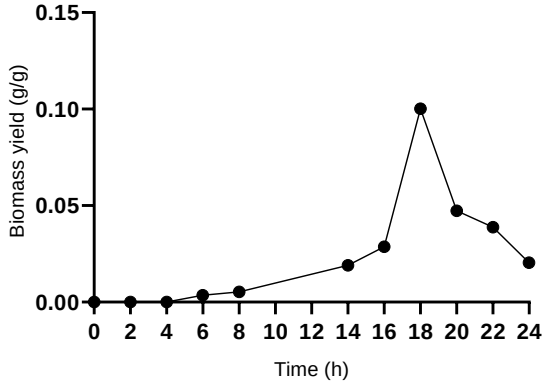


Figure 30: Biomass yield (Y X/S) vs fermentation time for rice flour fermentation with pH control.

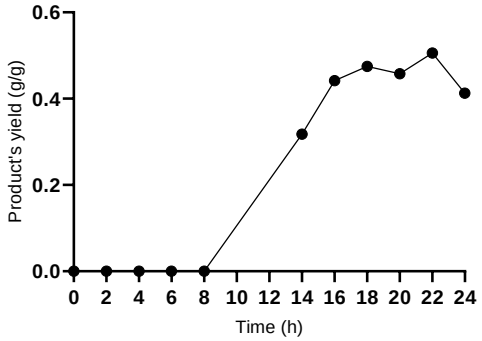


Figure 31: Product's yield (Y P/S) vs fermentation time for rice flour fermentations with pH control.

Table 18: Fermentation parameters: doubling time (td), constant growth rate (K), generation number (n), final biomass yield ($Y_{X/S}$) and Lactic acid yield ($Y_{P/S}$) for rice flour fermentation.

td (h)	K (h^{-1})	N	$Y_{X/S\ (t=24h)}$ (g/g)	$Y_{P/S(t=24h)}$ (g/g)
1.73	0.58	8.10	0.02	0.41

Monod's model was linearized by Hanes linearization model because Lineweaver-Burk's linearization gave unsatisfying R^2 result. Hanes Linearization was obtained applying the equation (10)

$$\frac{S}{\mu} = \frac{Ks}{\mu max} + \frac{S}{\mu max}$$

Hanes Linear regression provides a higher R^2 of 0.938 and the values obtained for K_s and μ_{max} are reported in table (19). Substrate's concentration (S) is higher than Ks during the exponential phase, indicating that the substrate's concentration satisfies the nutritional requirement of the microorganism.

Table 19: Kinetic constants of Monod's model, considering glucose as limiting substrate.

Ks (g/L)	μ_{max} (h^{-1})	R^2
2.79	0.45	0.938

Biomass and lactic acid productivities, W_X and W_P have been obtained according to Eq (11) and Eq (12) respectively. The dead time has been neglected. The maximum Wx is obtained after 18h of

fermentation with a value of 0.03 g/h corresponding to substrate consumption of 5.41 g/L. After which Wx begins to decrease until the end of the process (Figure 32).

Figure (33) reports the lactic acid productivity (W_P) referred to substrate consumption during the fermentation process.The maximum W_P is obtained after 22h of fermentation, with a value of 0.19 g/h corresponding to a substrate consumption S_{con} of 8.28g/L.

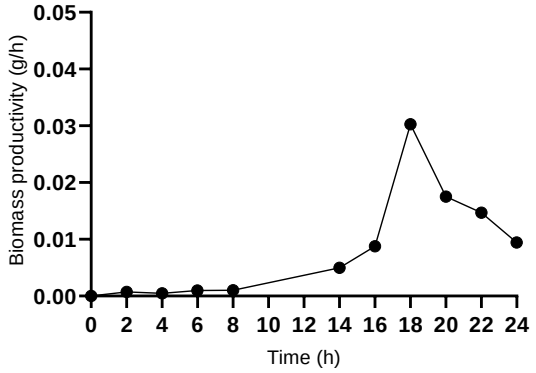


Figure 32:Biomass productivity vs fermentation time for rice flour fermentations with pH control.

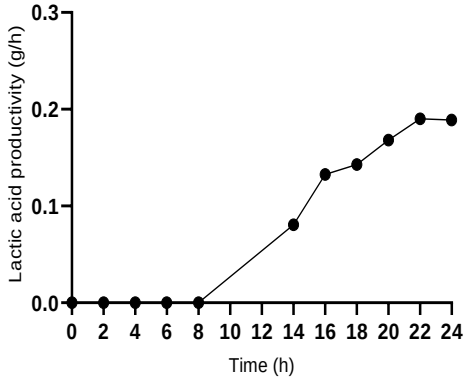


Figure 33: Lactic acid productivity vs fermentation time for rice flour fermentations with pH control.

4.1.3 Stabilization of fermented substrates and freeze-drying

Milk and rice flour fermented samples were taken at 24h and were stabilized through heat treatment (90°C/1 min) to reduce the bacterial

charge to lower than 10^2 CFU/mL. Samples were then freeze-dried for 24h and analyzed to investigate the effect of the treatments on lactic acid and active metabolite concentrations. Results provided that concentration of lactic acid and active metabolite were not affected by the stabilization treatment.

4.1.4 Properties of the active metabolite: trials with Caco-2 cells

To evaluate the effect of the postbiotic produced during rice flour fermentations, two cases were tested, using freeze-dried samples from two different rice flour fermenting conditions reported in section (3.3.3):

- Rice flour fermentations with no pH control
- Rice flour fermentation with pH control

It was then possible to investigate the efficiency of the biological effect taking into consideration different aspects. The use of two different trials; case 1 and case 2 allowed the simulation of two different conditions of the digestive system.

Case 1: It simulated the condition in which enterocytes absorb the fermented product and only after permeation they come into contact with the toxic peptide. Therefore, in this case, contact time with the cells is limited.

Case 2: the fermented substrate and the peptide coexist.

The comparison of the two cases is addressed to understand if there are differences in the administration of the postbiotic or in the time of contact.

The choice to use two different fermented samples instead, lies in the interest of highlighting a different result depending on the treatment to which the substrate was subjected.

The figures (34) through (36) report the immunofluorescence images of Caco-2 cells, the peptide (P31-43) is in red and the cell boundaries are marked in white.

Figure (34) shows the reference sample of Caco-2 cells stimulated only by the lissaminated peptide. Instead, images of the cells with the fermented product with “case 1” and “case 2” procedures are shown in figures (35) and (36).

Figure (35) shows images from “case 1” where Caco-2 cells were treated with fermented rice’s (A) with pH control and (B) without pH control respectively for 30 min then treated with P31-43-liss.

Figure (36) shows images from “case 2” where Caco-2 cells were treated for 30 min with fermented rice (A) with and (B) without pH control, washed with PBS and then treated with P31-43-liss.

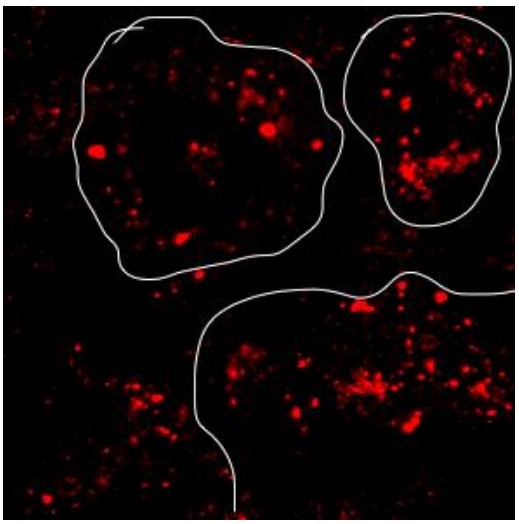


Figure 34: Map of the P31-43 and cell boundary. Caco-2 cells stimulated only by the lissaminated peptide.

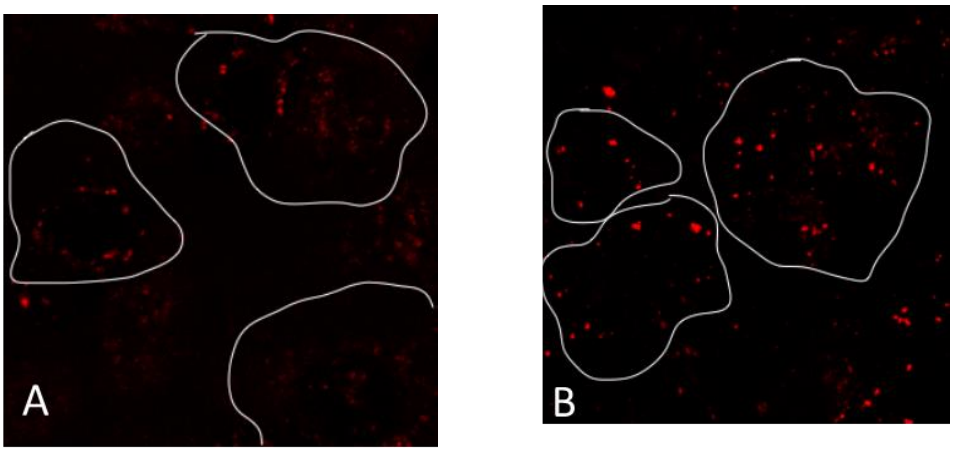


Figure 35: Images of Caco-2 cells treated according to case 1 with fermented rice and treated with P31-43-liss. (A) with pH control, (B) without pH control

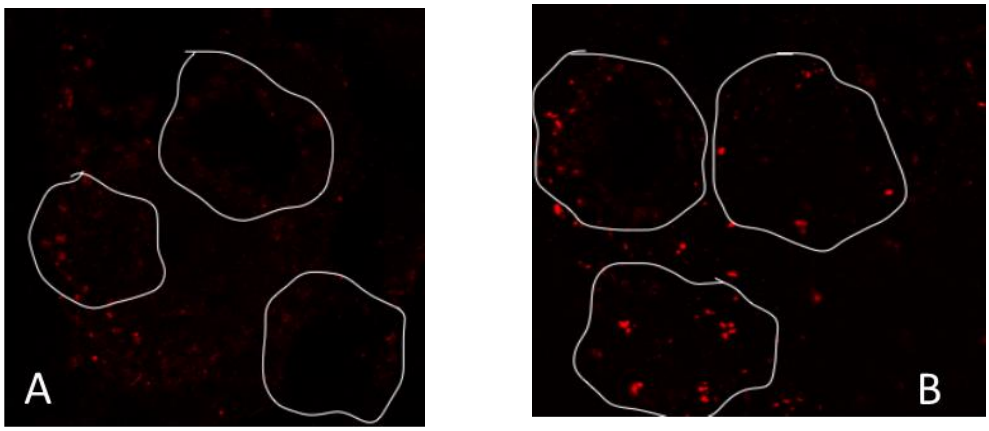


Figure 36: Images of Caco-2 cells treated according to case 2 with fermented rice, washed with PBS and then treated with P31-43-liss. (A) with pH control, (B) without pH control

In figure (37) the fluorescence intensity of the various cases is reported. According to this data, regarding the methods of administration of the postbiotic, there seems to be no difference between case 1 and case 2.

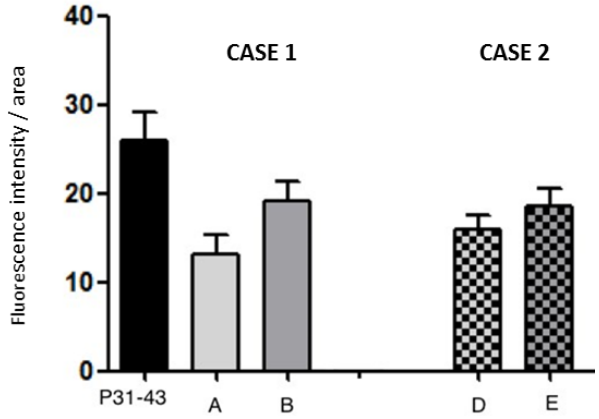


Figure 37: Statistical data of fluorescence intensity for case 1 and case 2. Case 1: with washing. A: pH controlled (-49%) B: no pH control (-26%). Case 2 (without washing) D: pH controlled (-40%) E: no pH control (-28%)

The comparison of results obtained with the two types of fermented substrate used points out that pH controlled process has a greater decrease in fluorescence intensity (49%) compared to an uncontrolled pH system (29%). It means that in the presence of the postbiotic product with fermentation at controlled pH, the entry of the toxic peptide into the cells is more hampered. Therefore, the product obtained has a better functionality.

4.1.5 Fermentations of supernatant of rice flour and water suspensions

The solid phase of the rice flour and water suspension was harvested by centrifugation and the liquid was used as broth characterized by low content of nutrients.

Centrifugation carried out under severe centrifugation (high centrifugation rotation rate) deprived the liquid phase of suspended nutrients to such an extension that no bacteria growth was recorded on the recovered liquid phase (not reported). According to this observation, the maximum centrifugation rate investigated was set at 2000 rpm.

Fermentation tests were carried out with broth produced by centrifugation under two centrifugal speeds: 2000 rpm and 1000 rpm both for 15 min. The broth produced at the two speeds were characterized by different nutrient content (total proteins and sugar) which led to different bacterial growth and production of lactic acid. The results of the fermentation for each broth are reported hereinafter.

4.1.5.1 Supernatant recovered at 1000 rpm for 15 min

The fermentation tests were carried out without pH control. Rice flour and water suspension centrifuged at 1000 rpm for 15 min gave the following results.

Fermentations started with mean pH value (t_0) at 5.27 ± 0.0 and dropped to 3.42 ± 0.0.

Figure (38) reports cell concentration vs. fermentation time. Fermentations started with an initial bacterial charge (t_0) of 2.52E+06 ± 5.68E+05 CFU/mL and ended with a charge (t_{24}) of 4.26E+07 ± 1.82E+07 CFU/mL with a maximum growth after 16h of fermentation (bacterial charge of 8.39E+07 ± 1.09E+07 CFU/mL).

The primary metabolite produced was lactic acid. Its concentration was measured as described before (2.4.2). Its production started at 16h of fermentation (t_{16}) with a value of 0.405 ± 0.002 g/L and a final concentration of 0.374 ± 0.002 g/L. The maximum production of lactic acid was around 20h of fermentation (0.486 ± 0.002 g/L).

Butyric, acetic and propionic acids were not found.Figure (39) reports organic acids concentrations vs. fermentation time.

Glucose quantity remained stable throughout the fermentation; initial quantity measured at t_0 was 2.09± 0.045 g/L and the final concentration measured at t_{24} was 2.3 ± 0.09 g/L.

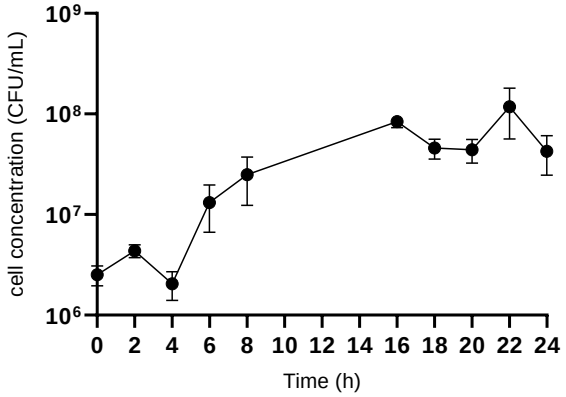


Figure 38: Cell concentration vs. fermentation time measured during fermentation of rice flour supernatant recovered by centrifugation at 1000 rpm/15 min. Fermentation performed without pH control.

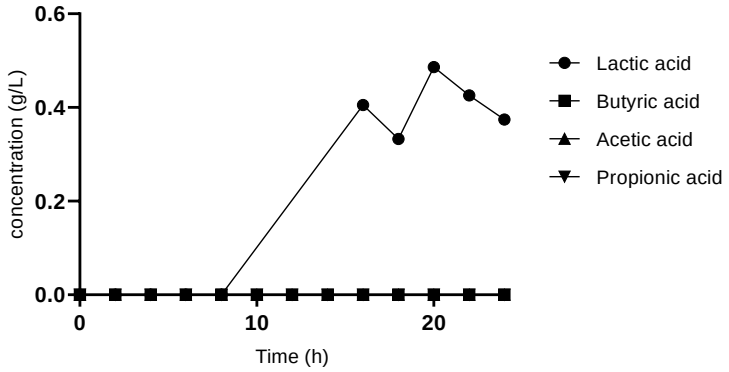


Figure 39:Organic acids concentrations vs. fermentation time measured during fermentation of rice flour supernatant recovered by centrifugation at 1000 rpm/15 min. Fermentation performed without pH control.

4.1.5.2 Supernatant recovered at 2000 rpm for 15 min

The fermentation tests were carried without pH control. Rice flour and water suspension centrifuged at 2000 rpm/15 min gave the following results:

These feremntations started with initial pH value (t0) at 5.23 ± 0.0 and dropped to 3.52 ± 0.0.

Figure (40) reports cell concentration vs. fermentation time. The fermentations started with an initial (t_0) bacterial charge of 2.88E+06 ± 6.03E+05 CFU/mL and ended (t_{24}) with a charge of 1.80E+07 ± 2.61E+06 CFU/mL. The maximum bacterial charge was registered at 16h of fermentation (t_{16}) with a concentration of 1.7E+07 ± 7.2E+06 CFU/mL.

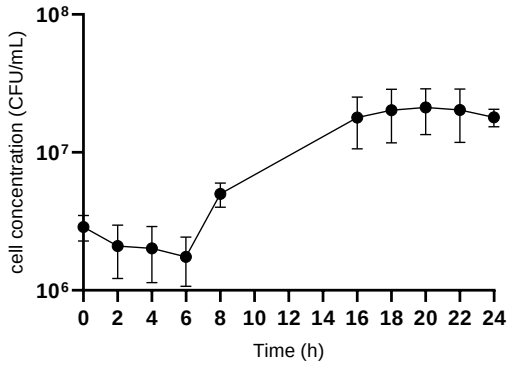


Figure 40: Cell concentration vs. fermentation time measured during fermentation of rice flour supernatant recovered by centrifugation at 2000 rpm/15 min. Fermentation performed without pH control

The primary metabolite produced during the fermentations was lactic acid. Its concentration was measured as described before (2.4.2). The production of lactic acid was measured at t_{16} (0.09 ± 0.002 g/L) and increased to 0.25 ± 0.002 g/L at 24h of fermentation (t_{24}), with a maximum concentration reached at t_{20} of 0.35 ± 0.004 g/L. Butyric, acetic and propionic acids were not found. Figure (41) reports organic acids concentration vs. fermentation time.

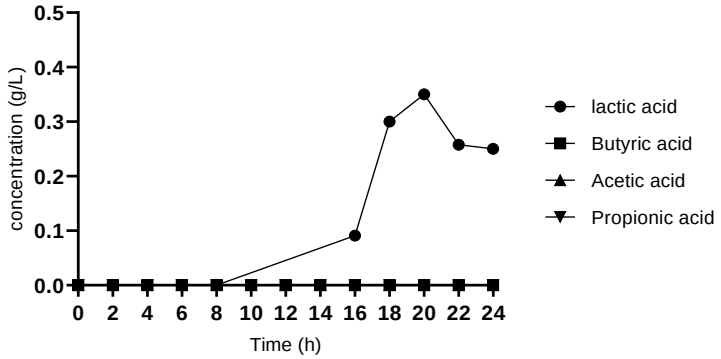


Figure 41: Organic acid concentrations vs. fermentation time measured during fermentation of rice flour supernatant recovered at 2000 rpm/15 min. Fermentation performed without pH control

Glucose quantities remained almost constant throughout the fermentation, the initial concentration measured was 2.31 ± 0.06 g/L whereas the quantity measured at the end of the fermentation was 2.09 ± 0.05g/L.

4.1.5.3 Analysis of the centrifugal harsh conditions on fermentation performance

The two supernatant's obtained from the two centrifugal speeds gave satisfying results in terms of bacterial growth and lactic acid production. Both fermenting conditions started with initial bacterial charge of 10^6 CFU/mL (2.52E+06 ± 5.68E+05 CFU/mL for 1000 rpm vs. 2.88E+06 ± 6.03E+06CFU/mL for 2000 rpm) and both reached the maximum growth at 16h with a higher charge for 1000rpm (8.39E+07 ± 1.09E+07CFU/mL for 1000 rpm vs. 1.7E+07 ± 7.2E + 06 CFU/mL for 2000 rpm). The amount of lactic acid produced was also higher for the 1000 rpm supernatant (0.489 ± 0.002 g/L) compared to the amount measured for 2000 rpm (0.35 ± 0.004 g/L).

The next step is to investigate the quantities of active metabolite produced by each condition, and test the biological properties.

4.2 Pilot Scale Results

Tests were carried out in Nijmegen, the Netherlands. Fermentation tests were performed following the protocol, fermentation system and recipe described in section (3.8.2) (3.7) (3.6.2) respectively. The fermentations were carried out in 200 L reactor. Tests lasted five days between preparations and the fermentation process in itself. Samples were withdrawn from a tap aseptically as described in section (3.7). Main results obtained for milk and rice flour fermentations are reported hereinafter in terms of bacterial growth, lactic acid production, active compounds' production and effect of drying techniques on the broth

4.2.1 Milk fermentation

Fermentation tests were carried out with pH control set at 6.2 and maintained by feeding NaOH (30% w/v) solution by means of a peristaltic pump.

In figure (42) the growth curve is reported. A lag phase of 2 hours, and exponential phase of 16h (t_{18}). The stationary phase started after 20h until the end of the process. Fermentations started with an initial bacterial charge of 1.12E+06 ± 7.4E+05 CFU/mL, reached a maximum charge of 6.73E+08 ± 2.6E+08 CFU/mL and a final charge (t_{24}) of 5.53E+08 ±1.5E+08 CFU/mL.

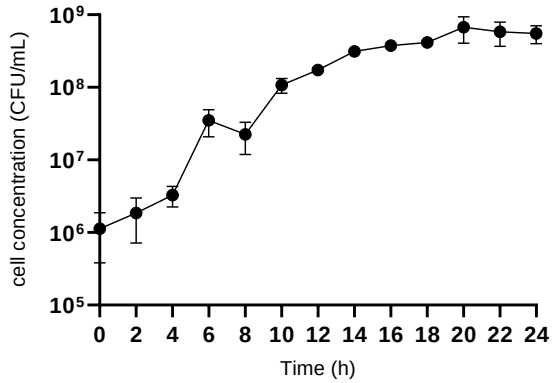


Figure 42: Cell concentration vs. fermentation time measured during milk fermentations under pH control.

The primary metabolite produced during the fermentations was Lactic acid. Its concentration was measured as described in section (3.9.2). Figure (43) reports lactic acid concentration vs. fermentation time. Its concentration increased during the fermentation to a maximum of 2.105 ± 0.25 g/L at 24h.

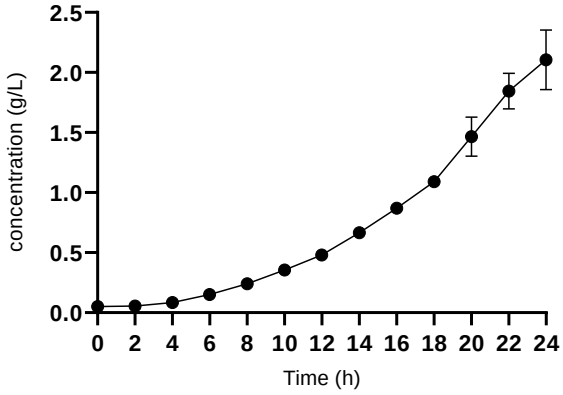


Figure 43: Lactic acid concentration vs. fermentation time measured during milk fermentations under pH control.

Figure (44) shows the accumulation curve of the active metabolite in time. During milk fermentations the active metabolite was detected at 16h of fermentation (t_{16}) with 8.8 ± 1.9 mg/L and reached a maximum of 13.7 ± 1.8 mg/L at 24h (t_{24}).

The fermented suspension was pasteurized (heat treated to stabilize the product) according to the protocol reported in section (3.8.2); The pasteurization completely reduced the bacterial charge and slightly

impacted lactic acid and active metabolite's concentrations. The lactic acid and active metabolite's concentration reduced to 1.96 g/L and 11.33 mg/L, respectively.

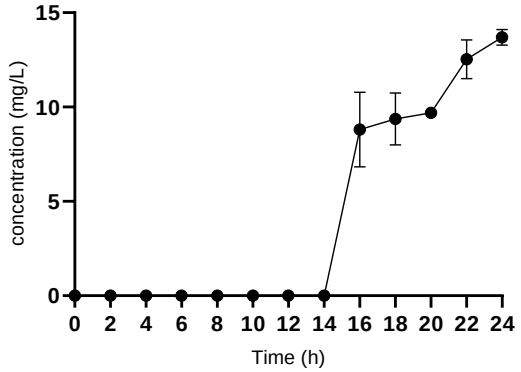


Figure 44: Active metabolite's accumulation vs. fermentation time measured during milk fermentations under pH control.

After pasteurization, the broth was dried through spray drying according to the protocol reported in section (3.8.2). Spray drying had no effect on lactic acid and active metabolite concentrations. Table (20) reports the bacterial charge, lactic acid and active metabolite concentrations at the end of fermentation, after pasteurization and after spray drying.

Table 20: Bacterial charge, lactic acid and active metabolite's concentrations at the end of fermentation and after pasteurization and spray drying.

	Bacterial charge CFU/mL	Lactic acid g/L	Active metabolite mg/L
T24	5.53E+08	2.01	13.7
Pasteurized product	0.00E+00	1.96	11.3
Spray dried product	0.00E+00	1.96	10.8

4.2.2 Rice flour fermentation

Fermentation tests were carried out with pH control set at 6.2 and maintained by feeding NaOH (30% w/v) solution by means of a peristaltic pump.

In figure (45) the growth curve is shown. The lag phase is not detected, an exponential phase starts almost immediately and lasts for 16h. The stationary phase started after 16h and lasted until the end of the fermentation. The initial bacterial charge (t_0) of 8.25E+05 ± 1.7E+05 CFU/mL with a final charge of 1.45E+09 ± 2.47E+08 CFU/mL.

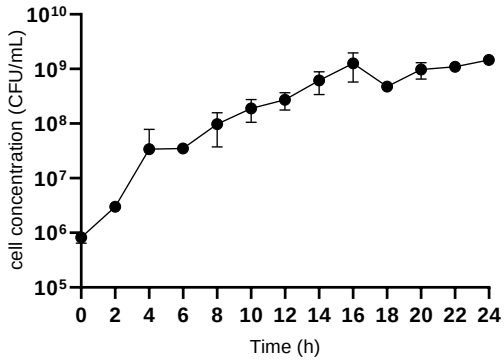


Figure 45: Cell concentration vs. fermentation time measured during rice flour fermentation carried out with pH control.

The primary metabolite produced during the fermentations was lactic acid. Its concentration was measured as described in section (3.9.2). The production of lactic acid increased during the fermentation to 6.33 ± 0.74 g/L at 24h (t_{24}). Figure (46) reports lactic acid concentration vs. fermentation time.

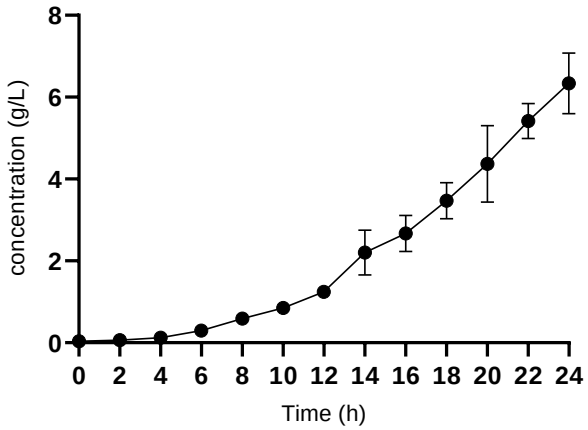


Figure 46: Lactic acid concentration vs. fermentation time measured during rice flour fermentation carried out with pH control.

Figure (47) reports the accumulation of active metabolite in time. During rice flour fermentations, the active metabolite was measured after14h (t_{14}) of fermentation reaching 22.2 ± 0.88 mg/L at the end of the process (t_{24}).

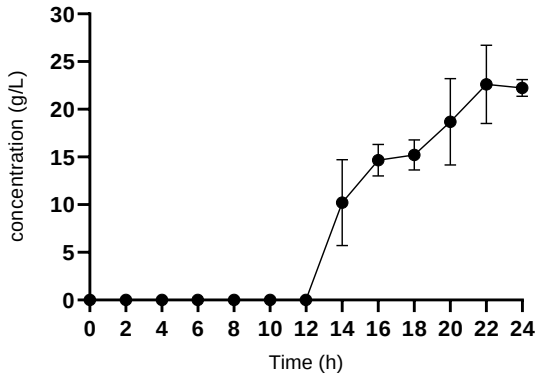


Figure 47: Active metabolite concentration vs. fermentation time measured during rice flour fermentation carried out with pH control.

The fermented suspension was pasteurized (heat treated to stabilize the product) according to the protocol reported in section (3.8.2). The pasteurization completely reduced the bacterial charge and slightly impacted on lactic acid and active metabolites's concentrations. The lactic acid and active metabolites reduced to 5.5 g/L and 21.7 mg/L, respectively.

After pasteurization, the broth was dried through spray drying according to the protocol reported in section (3.8.2). Spray drying had a slight effect on lactic acid and active metabolite concentrations reduced to 4.9 g/L and 19.7 mg/L. Table (21) reports the bacterial charge, lactic acid and active metabolite concentrations at the end of the fermentation, after pasteurization and after spray drying.

Table 21: Bacterial charge, lactic acid and active metabolite's concentrations at the end of fermentation and after pasteurization and spray drying.

	Bacterial charge CFU/mL	Lactic acid g/L	Active metabolite mg/L
T24	1.45E+09	6.33	22.2
Pasteurized product	0.00E+00	5.5	21.7
Spray dried product	0.00E+00	4.9	19.7

4.2.3 Shelf life analysis

Spray dried samples from the pilot plant were stored at three different temperatures (4°C, 20°C and 37°C) to see the effect of these conservation temperatures on the product and its characteristics.

Spray dried milk samples were analyzed in terms of lactic acid and active metabolite's concentrations. The starting quantities were of 2.5 g/L and 8.8 mg/L for lactic acid and active metabolite respectively.

Different temperatures seem to have the same effect on the samples. Results are reported in table (22) for lactic acid and table (23) for the active metabolite.

Table 22: Spray dried fermented milk shelf life analysis lactic acid g/L

	4°C	***20°C***	***37°C***
Month	***Lactic acid g/L***		
1	1.73	1.73	1.77
2	1.64	1.75	1.6
3	1.9	1.98	1.68
5	1.28	1.23	1.27

Table 23: Spray dried fermented milk shelf life analysis active metabolite

	4°C	***20°C***	***37°C***
Month	***Active metabolite mg/L***		
1	9	9	9.2
2	10.4	10.8	9.4
3	9.4	9.6	9.8
5	9.8	8.8	9.3

Regarding rice samples, the starting quantity of lactic acid was 5.5 g/L and 19.7 mg/L active metabolite. Analysis done on these samples showed no great effect on lactic acid's concentration (table 24), and a slight decrease in active metabolite's quantity. (Table 25)

Table 24: Spray dried fermented rice samples shelf life analysis lactic acid

	4°C	***20°C***	***37°C***
Month	***Lactic acid g/L***		
1	4.7	4.9	5.8
2	5.9	6.2	5.6
3	5.5	5.7	5.3
4	5.5	5.4	5.3
6	4.5	3.5	3.9

Table 25: Spray dried fermented rice samples shelf life analysis active metabolite (mg/L)

	4°C	***20°C***	***37°C***
Month	***Active metabolite mg/L***		
1	19.1	19.2	20
2	19.18	19.7	18.2
3	16.5	16.2	15.9
4	18.9	16.9	18
6	19.2	18.6	18.6

4.3 Analysis of the process scale-up on product features

Other than the differences in terms of volume, apparatus, trial duration and preparation process, the scaling of the process from laboratory scale to pilot scale was efficient.
Reported here is a comparison between lab and pilot scales main results.

4.3.1 Milk fermentations

Bacterial growth followed the same trend in both scales. Figure (48) reports the cell concentration vs. fermentation time. Both processes presented a 2h lag phase, 18h exponential phase and a stationary phase that started after 20h of fermentation.

The maximum bacterial charge registered at lab scale was 1.66E+08 ± 3.35E+08 CFU/mL at 14h of fermentations whereas the maximum growth was 6.7E+08 ± 2.65E+08 CFU/mL registered at 18h at pilot scale.

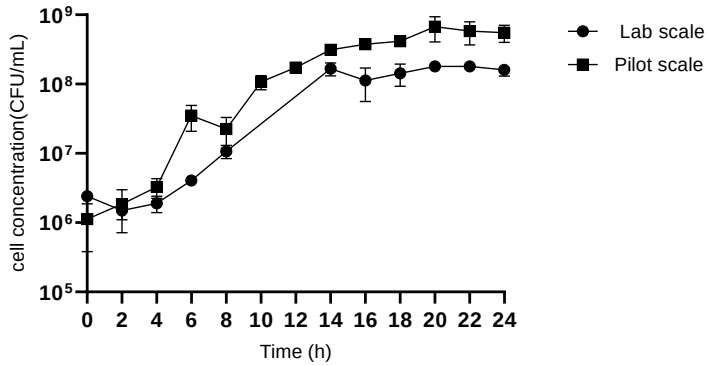


Figure 48: Cell concentration vs. fermentation time for lab and pilot scale. Milk fermentations carried out with pH control.

Figure (49) reports lactic acid concentration vs. fermentation time for both scales. The highest concentration of lactic acid at lab scale was measured at 1.3 ± 0.0 g/L at lab scale (t_{24}) and 2.10 ± 0.25 g/L at pilot scale (t_{24}). The quantity of lactic acid measured in both scales is low for this kind of lactic fermentations which is due to the fermentation conditions implemented.

Figure (50) reports the active metabolite concentration vs. fermentation time for both scales.The highest concentration of active metabolite was registered at 24h (t_{24}) for both scales with a concentration of 6.9 ± 0.5 mg/L at lab scale and 13.7 ± 0.42 mg/L at pilot scale.

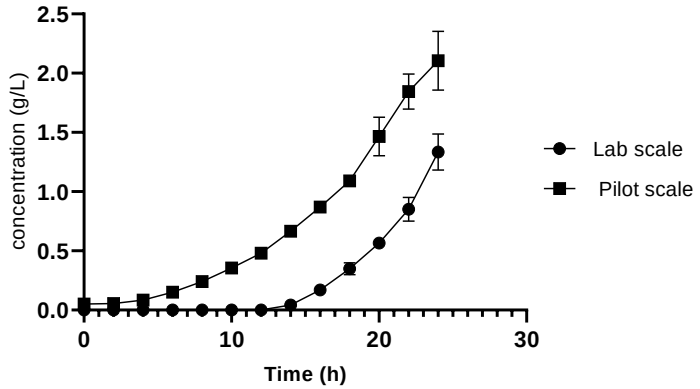


Figure 49: Lactic acid concentration vs. fermentation time for lab and pilot scales. Milk fermentations carried out with pH control.

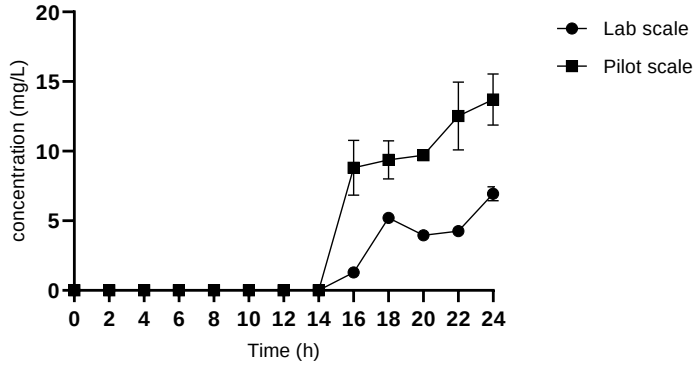


Figure 50: Active metabolite concentration vs. fermentation time for lab and pilot scales. Milk fermentations carried out with pH control.

4.3.2 Rice fermentations

Bacterial growth also followed the same trend at both scales. Figure (51) reports cell concentration vs. fermentation time for lab and pilot

scales. The process at lab scale presented a lag phase of 4h, an exponential phase of 14h and a stationary phase that started after 18h of fermentation. On the other hand, pilot scale fermentations presented a very short lag phase undetectable through our analysis, an exponential phase of 14h and a stationary phase that started after 16h of fermentation. The maximum bacterial charge registered was 5.45E+08 ± 1.47E+08 CFU/mL at lab scale (t18) and 1.45E+09 ± 2.47E+08 CFU/mL at pilot scale (t24).

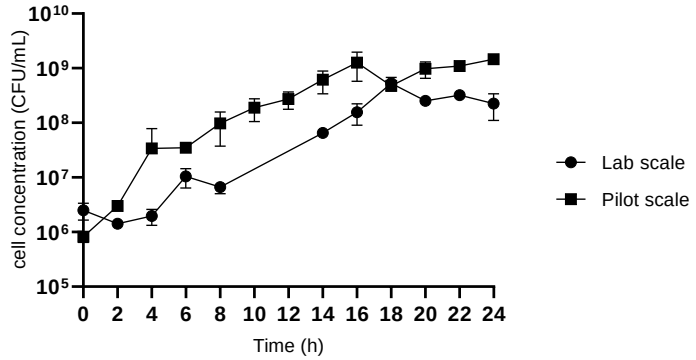


Figure 51: Cell concentration vs. fermentation time for lab and pilot scales. Rice flour fermentations carried out with pH control.

Figure (52) reports lactic acid concentration vs. fermentation time for lab and pilot scales. The maximum concentration of lactic acid registered at pilot scale was 4.4 ± 0.2 g/L measured at 24h (t24) while, at pilot scale, the maximum concentration was also measured at 24h 6.33 ± 0.7 g/L. The quantity of lactic acid measured in both scales is low for this kind of lactic fermentations which is due to the fermentation conditions implemented.

Figure (53) reports active metabolite concentration vs. fermentation time for lab and pilot scales. The maximum concentration measured at lab scale was 17.6 ± 0.35 mg/L (t_{24}) and at pilot scale 22.2 ± 0.8 mg/L (t_{24}).

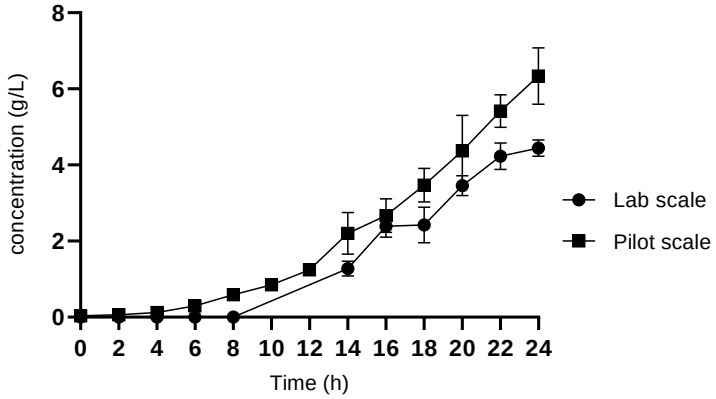


Figure 52: Lactic acid concentration vs. fermentation time for lab and pilot scales. Rice flour fermentations were performed with pH control.

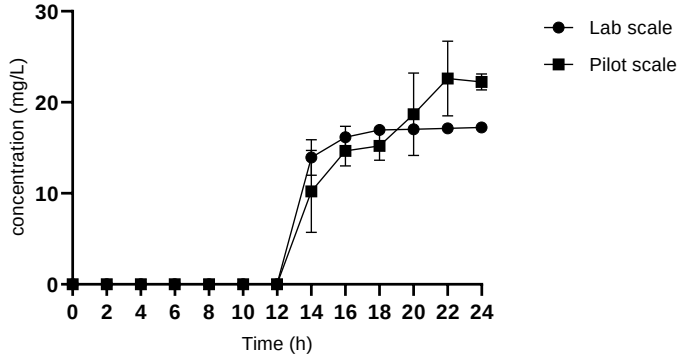


Figure 53: Active metabolite concentration vs. fermentation time for lab and pilot scales. Rice flour fermentations were performed with pH control.

Both feedstocks (milk and rice flour) presented the same trend of bacterial growth, lactic acid production and active metabolite production at lab and pilot scales. This suggests the effiecieny of the scaling up from the lab scale (1.5 L) to the pilot scale (200 L).

Conclusions

Lactic acid fermentation of milk and rice flour using the probiotic *L. paracasei CBA L74* was used to produce postbiotic molecules with beneficial effect. Fermentation tests were successfully carried out at lab scale and pilot scale.

Lab scale permitted the development of an adequate recipe and protocol for the feedstocks chosen. Fermentation steps are represented in the schematic flow chart. Figure (54)

The fermentation feedstocks were: milk, rice flour and water, and supernatant of rice flour recovered after centrifugation.

- Milk- a highly consumed food around the world. Two conditions were tested during milk fermentations: with and without pH control, to verify the effect of such parameter on bacterial growth and lactic acid.

Main results obtained showed that fermentations with pH control represented a better condition, both for bacterial charge (without pH control: T_{24} charge registered 2.8E+08 ± 4.9E+07 CFU/mL vs 1.6E+08 ± 3E+07 CFU/mL with pH control) and lactic acid production (3.5 ± 0.05 g/L without pH control vs 1.3 ± 0.0 g/L) when compared to the results obtained when pH wasn't controlled.

- Rice flour: Two conditions were tested at lab scale: fermentations with and without pH control. Rice flour fermentations carried out under pH control presented high bacterial growth and production of lactic acid (with pH control: 5.3E+08 ± 1.47E + 08 CFU/mL reached at 18 h vs 5.2E+08 ± 9.1E+07 CFU/mL without pH control reached at 24h and 4.4± 0.21 g/L with pH control vs 1.9 ± 0.07 g/L without pH control).

Analysis to quantify the active metabolite (postbiotic) produced during milk and rice flour fermentations was only performed for fermentations executed under pH control. Its quantities were higher in rice flour (17.23 ± 0.35mg/L) fermentations when compared to milk fermentations (6.9 ± 0. 5 mg/L).

The beneficial activity induced by fermentation metabolite(s) was proven via trials performed on Caco-2 cells, effecting the entrance of gliadin peptide P31-43 into the cells. The cell free supernatant of rice flour obtained in two conditions- with and without pH control- interfered in the transport of P31-43 in different measure. pH controlled cell free supernatant interfered more efficiently respect to cell free supernatant of rice flour fermented without pH control.

- Rice flour supernatants: Trials to test the possibility of fermenting *L. paracasei CBA L74* on minimal matrix obtained from rice flour and water suspension were also performed. The aim to select a minimal

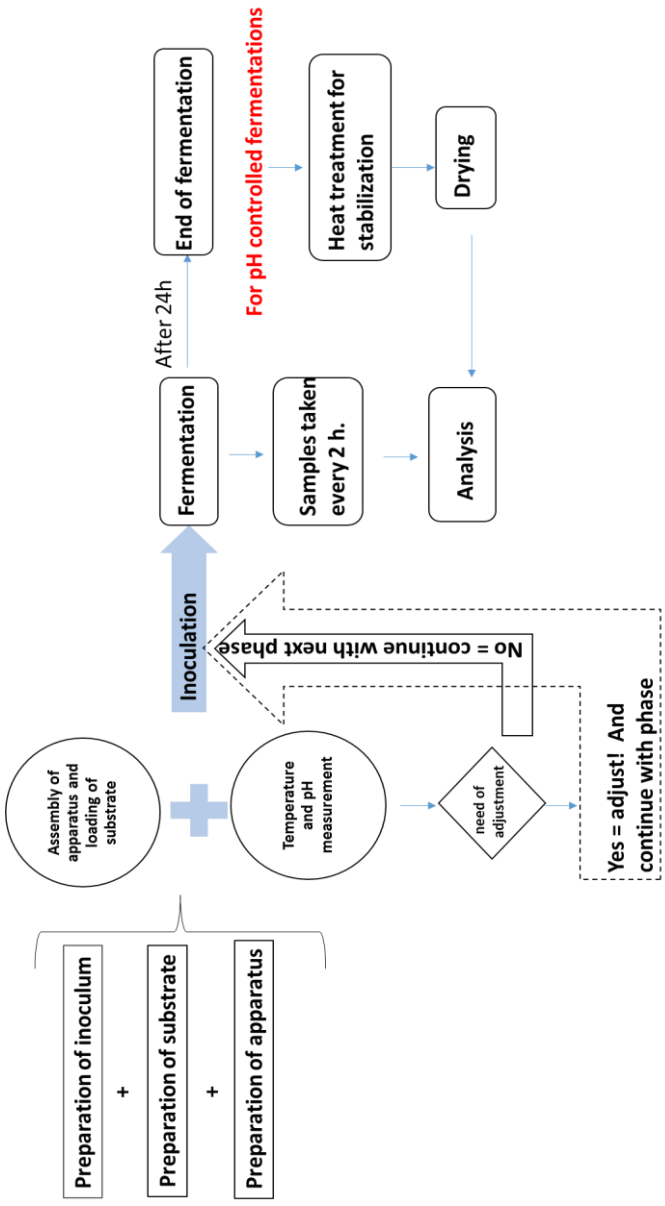
broth characterized by low viscosity- close to that of the water- and smaller than that of the rich broth- was to save energy for mixing, as well as simplify the analysis. The low viscosity broths were obtained through centrifugation of the initial rice flour and water suspension. Different centrifugal speed caused different separation between the solid particles and liquids. High centrifugal speed deprived the matrix of nutrients to such an extent that no microbial growth or metabolite production were registered. This limited the speed to which the suspension was submitted and two conditions were tested: fermentation on rice flour suspension obtained from centrifugation at 1000 rpm/15 min and after centrifugation at 2000 rpm/15 min. As expected, the lower centrifugal speed (1000 rpm/15 min) provided a suspension with a high amount of nutrients when compared to the other speed (2000 rpm/15 min) which resulted in high growth and production of lactic acid at 24h (4.26E + 07 ± 1.82E +07 CFU/mL and 0.374 ±0.002 g/L for 1000 rpm/15min vs 1.80E+07 ± 2.61E+06 CFU/mL and 0.25± 0.002 g/L for 2000 rpm/15 min). The next steps for these fermentations is the quantification of the active metabolite produced and verifying its effects.

The scale up of the entire process to the pilot scale demonstrated an efficient scaling up. These fermentations were carried under pH control. Results obtained in terms of bacterial growth, lactic acid and active metabolite productions for each feedstock were comparable to those obtained at lab scale:

- Milk: these fermentation presented a maximum cell concentration measured at t_{18} of 6.7E + 08 ± 2.65E+08 CFU/mL. The concentration of Lactic acid and active metabolite measured at 24h of fermentation were 2.1 ± 0.25 g/L and 13.7±0.42 mg/L respectively.
- Rice flour: these fermentaions presented a maximum cell concentration ar t_{24}. The concentrations of lactic acid and active metabolite measured at 24h were 6.33 ± 0.7 g/L and 22.2 ± 0.8 mg/L respectively.

In conclusion, this project aimed to develop the processes necessary for the production of postbiotic functional foods through fermentation of a probiotic with proven beneficial effects.[74]–[76]. The feedstocks chosen for the fermentation represent a staple food for the majority of the world's population, furthermore, both have good nutritional value. Like other LAB, *L. paracasei CBA L74* underwent lactic acid fermentation to produce lactic acid and postbiotic molecule(s). The detection of the postbiotic took place through analysis of the fermented matrix using the MALDI-TOF then HPLC. The positive effect tested *in-vitro* on Caco-2 cells.

Figure 54: fermentations flow chart



Reference

[1] A. Blandino, M. E. Al-Aseeri, S. S. Pandiella, D. Cantero, and C. Webb, “Cereal-based fermented foods and beverages,” *Food Res. Int.*, vol. 36, no. 6, pp. 527–543, 2003.

[2] G. Hardy, “Nutraceuticals and functional foods: introduction and meaning,” *Nutrition*, vol. 16, no. 7–8, pp. 688–689, 2000.

[3] C. Stanton, R. P. Ross, G. F. Fitzgerald, and D. Van Sinderen, “Fermented functional foods based on probiotics and their biogenic metabolites,” *Curr. Opin. Biotechnol.*, vol. 16, no. 2, pp. 198–203, 2005.

[4] N. S. Kwak and D. J. Jukes, “Functional foods. Part 1: The development of a regulatory concept,” *Food Control*, vol. 12, no. 2, pp. 99–107, 2001.

[5] M. E. Sanders, “Overview of functional foods: Emphasis on probiotic bacteria,” *Int. Dairy J.*, vol. 8, no. 5–6, pp. 341–347, 1998.

[6] I. Siró, E. Kápolna, B. Kápolna, and A. Lugasi, “Functional food. Product development, marketing and consumer acceptance--a review.,” *Appetite*, vol. 51, no. 3, pp. 456–67, 2008.

[7] E. Isolauri, “Probiotics in human disease 1–3,” *Am. Soc. Clin. Nutr.*, vol. 73, no. April 2000, pp. 1142–1146, 2001.

[8] H. in’t V. J. Holzapel WH, Haberer P, Snel J, Schillinger U, “Overview of gut flora and probiotics. Intl J Food Microbiol,” vol. 41, pp. 85–101, 1998.

[9] R. Fuller, “Probiotics in human medicine.,” *Gut*, vol. 32, no. 4, pp. 439–442, 1991.

[10] R. George Kerry, J. K. Patra, S. Gouda, Y. Park, H. S. Shin, and G. Das, “Benefaction of probiotics for human health: A review,” *J. Food Drug Anal.*, vol. 26, no. 3, pp. 927–939, 2018.

[11] M. E. Sanders, “Summary of Conclusions from a Consensus Panel of Experts on Health Attributes of Lactic Cultures: Significance to Fluid Milk Products Containing Cultures,” *Journal of Dairy Science*, vol. 76, no. 7. pp. 1819–1828, 1993.

[12] M. Kaila, E. Isolauri, E. Soppi, E. Virtanen, S. Laine, and H. Arvilommi, “Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain,” *Pediatr. Res.*, vol. 32, no. 2, pp. 141–144, 1992.

[13] E. J. Schiffrin, F. Rochat, H. Link-Amster, J. M. Aeschlimann, and A. Donnet-Hughes, “Immunomodulation of Human Blood Cells Following the Ingestion of Lactic Acid Bacteria,” *J. Dairy Sci.*, vol.

78, no. 3, pp. 491–497, 1995.
[14] C. Maldonado Galdeano, A. De Moreno De Leblanc, G. Vinderola, M. E. Bibas Bonet, and G. Perdigón, “Proposed model: Mechanisms of immunomodulation induced by probiotic bacteria,” *Clin. Vaccine Immunol.*, vol. 14, no. 5, pp. 485–492, 2007.
[15] S. Weston, A. Halbert, P. Richmond, and S. L. Prescott, “Effects of probiotics on atopic dermatitis: A randomised controlled trial,” *Arch. Dis. Child.*, vol. 90, no. 9, pp. 892–897, 2005.
[16] E. Songisepp *et al.*, “A new probiotic cheese with antioxidative and antimicrobial activity,” *J. Dairy Sci.*, vol. 87, no. 7, pp. 2017–2023, 2004.
[17] K. Hirayama and J. Rafter, “The role of probiotic bacteria in cancer prevention,” *Microbes Infect.*, vol. 2, no. 6, pp. 681–686, 2000.
[18] K. E. Scholz-Ahrens *et al.*, “Prebiotics, Probiotics, and Synbiotics Affect Mineral Absorption, Bone Mineral Content, and Bone Structure,” *J. Nutr.*, vol. 137, no. 3, pp. 838S-846S, 2007.
[19] D. I. A. Pereira and G. R. Gibson, “Effects of consumption of probiotics and prebiotics on serum lipid levels in humans,” *Crit. Rev. Biochem. Mol. Biol.*, vol. 37, no. 4, pp. 259–281, 2002.
[20] S. K. Yeo and M. T. Liong, “Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics,” *Int. J. Food Sci. Nutr.*, vol. 61, no. 2, pp. 161–181, 2010.
[21] R. F. AFRC, “Probiotics in man and animals,” *J. Appl. Bacteriol.*, vol. 66, no. 5, pp. 365–378, 1989.
[22] FAO & WHO, “Probiotics in food FOOD AND NUTRITION,” in *FAO Food and Nutrition Paper*, 2002.
[23] A. Cencic and W. Chingwaru, “The role of functional foods, nutraceuticals, and food supplements in intestinal health,” *Nutrients*, vol. 2, no. 6, pp. 611–625, 2010.
[24] M. E. Sanders, “How Do We Know When Something Called ‘ Probiotic ’ Is Really a Probiotic ? A Guideline for Consumers and Health Care Professionals,” *Funct. Food Rev.*, vol. 1, no. 1, pp. 3–12, 2009.
[25] M. K. Tripathi and S. K. Giri, “Probiotic functional foods: Survival of probiotics during processing and storage,” *J. Funct. Foods*, vol. 9, no. 1, pp. 225–241, 2014.
[26] S. López-Varela, M. González-Gross, and A. Marcos, “Functional foods and the immune system: A review,” *Eur. J. Clin. Nutr.*, vol. 56, pp. S29–S33, 2002.

[27] S. H. Al-Sheraji, A. Ismail, M. Y. Manap, S. Mustafa, R. M. Yusof, and F. A. Hassan, “Prebiotics as functional foods: A review,” *J. Funct. Foods*, vol. 5, no. 4, pp. 1542–1553, 2013.
[28] L. J. Fooks, R. Fuller, and G. R. Gibson, “Prebiotics , probiotics and human gut microbiology,” vol. 9, pp. 53–61, 1999.
[29] K. R. Pandey, S. R. Naik, and B. V. Vakil, “Probiotics, prebiotics and synbiotics- a review,” *J. Food Sci. Technol.*, vol. 52, no. 12, pp. 7577–7587, 2015.
[30] J. I. G. Ruth E ley, Peter J. Turnbaugh, Samuel Klein, “Human gut microbes associated with obesity,” *Nat. Microb. Ecol.*, vol. 444, no. 7122, pp. 1021–1022, 2006.
[31] S. A. Abrams *et al.*, “A combination of prebiotic short- and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents,” *Am. J. Clin. Nutr.*, vol. 82, no. 2, pp. 471–476, 2005.
[32] O. Chonan, R. Takahashi, and M. Watanuki, “Role of activity of astrointestinal microflora in absorption of calcium and magnesium in rats fed β1-4 linked galactooligosaccharides,” *Biosci. Biotechnol. Biochem.*, vol. 65, no. 8, pp. 1872–1875, 2001.
[33] M. Gmeiner *et al.*, “Influence of a synbiotic mixture consisting of Lactobacillus acidophilus 74-2 and a fructooligosaccharide preparation on the microbial ecology sustained in a simulation of the human intestinal microbial ecosystem (SHIME reactor),” *Appl. Microbiol. Biotechnol.*, vol. 53, no. 2, pp. 219–223, 2000.
[34] T. Asahara, K. Nomoto, K. Shimizu, M. Watanuki, and R. Tanaka, “Increased resistance of mice to salmonella enterica serovar typhimurium infection by synbiotic administration of bifidobacteria and transgalactosylated oligosaccharides,” *J. Appl. Microbiol.*, vol. 91, no. 6, pp. 985–996, 2001.
[35] M. Malaguarnera *et al.*, “Bifidobacterium longum with fructo-oligosaccharides in patients with non alcoholic steatohepatitis,” *Dig. Dis. Sci.*, vol. 57, no. 2, pp. 545–553, 2012.
[36] M. Sharma and G. Shukla, “Metabiotics: One step ahead of probiotics; an insight into mechanisms involved in anticancerous effect in colorectal cancer,” *Frontiers in Microbiology*, vol. 7, no. DEC. Frontiers Research Foundation, 2016.
[37] A. Amaretti, M. Nunzio, A. Pompei, S. Raimondi, and M. Rossi, “Antioxidant properties of potentially probiotic bacteria : in vitro and in vivo activities,” pp. 809–817, 2013.
[38] C. Ou, J. Ko, and M. Lin, “Antioxidative Effects of Intracellular Extracts of Yogurt Bacteria on Lipid Peroxidation and Intestine

407 Cells," *J. food*, vol. 14, no. September 2006, pp. 304–310, 2014.
[39] M. Sarno *et al.*, "Lactobacillus paracasei CBA L74 interferes with gliadin peptides entrance in Caco-2 cells," *Int. J. Food Sci. Nutr.*, vol. 65, no. 8, pp. 953–959, 2014.
[40] K. Y. Kareem, F. H. Ling, L. T. Chwen, O. M. Foong, and S. Anjas Asmara, "Inhibitory activity of postbiotic produced by strains of Lactobacillus plantarum using reconstituted media supplemented with inulin," *Gut Pathog.*, vol. 6, no. 1, pp. 1–7, 2014.
[41] R. Campana, S. Federici, E. Ciandrini, and W. Baffone, "Antagonistic activity of lactobacillus acidophilus ATCC 4356 on the growth and adhesion/invasion characteristics of Human Campylobacter jejuni," *Curr. Microbiol.*, vol. 64, no. 4, pp. 371–378, 2012.
[42] Y. Wang, "Prebiotics: Present and future in food science and technology," *Food Res. Int.*, vol. 42, no. 1, pp. 8–12, 2009.
[43] F. Yan, H. Cao, T. L. Cover, R. Whitehead, M. K. Washington, and D. B. Polk, "Soluble Proteins Produced by Probiotic Bacteria Regulate Intestinal Epithelial Cell Survival and Growth," *Gastroenterology*, vol. 132, no. 2, pp. 562–575, 2007.
[44] M. V. Tejada-simon, "Proinflammatory Cytokine and Nitric Oxide Induction in Murine Macrophages by Cell Wall and Cytoplasmic Extracts of Lactic Acid Bacteria," vol. 62, no. 12, pp. 1435–1444, 1999.
[45] H. Sokol *et al.*, "Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 43, pp. 16731–16736, 2008.
[46] A. Cicenia, A. Scirocco, and C. Severi, "Postbiotic Activities of Lactobacilli - derived Factors," *J. Clin. Gastroenterol.*, vol. 48, no. December, pp. 18–22, 2014.
[47] C. Klemashevich, C. Wu, D. Howsmon, R. C. Alaniz, K. Lee, and A. Jayaraman, "Rational identification of diet-derived postbiotics for improving intestinal microbiota function," *Curr. Opin. Biotechnol.*, vol. 26, pp. 85–90, 2014.
[48] R. D. Pridmore, A. C. Pittet, F. Praplan, and C. Cavadini, "Hydrogen peroxide production by Lactobacillus johnsonii NCC 533 and its role in anti-Salmonella activity," *FEMS Microbiol. Lett.*, vol. 283, no. 2, pp. 210–215, 2008.
[49] D. Fayol-messaoudi, V. Lie, and A. L. Servin, "pH-, Lactic Acid-, and Non-Lactic Acid-Dependent Activities of Probiotic Lactobacilli against," *Appl. Environ. Microbiol.*, vol. 71, no. 10, pp. 6008–

6013, 2005.
[50] F. Leroy and L. De Vuyst, “Lactic acid bacteria as functional starter cultures for the food fermentation industry,” *Trends Food Sci. Technol.*, vol. 15, no. 2, pp. 67–78, 2004.
[51] S. Muñoz-Quezada *et al.*, “Competitive inhibition of three novel bacteria isolated from faeces of breast milk-fed infants against selected enteropathogens,” *Br. J. Nutr.*, vol. 109, no. SUPPL. 2, 2013.
[52] Y. Wang, Y. Liu, A. Sidhu, Z. Ma, C. Mcclain, and W. Feng, “Lactobacillus rhamnosus GG culture supernatant ameliorates acute alcohol-induced intestinal permeability and liver injury,” *Am. J. gastointestinal liver Physiol.*, vol. 303, no. 1, pp. 32–41, 2012.
[53] I. Robles-vera, M. Toral, M. Romero, and R. Jiménez, “Antihypertensive Effects of Probiotics,” 2017.
[54] J. E. Aguilar-Toalá *et al.*, “Postbiotics: An evolving term within the functional foods field,” *Trends Food Sci. Technol.*, vol. 75, no. June 2017, pp. 105–114, 2018.
[55] and L. P. Sokol H., Pigneur B., Watterlot L., Lakhdari O., Bermudez-Humara L.G. , Gratadoux J., Blugeon S., Bridonneau C., Furet J., Corthier G., Grangette C., Vasquez N., Pochart P., Trugnan G., Thomas G., Blottiere H.M., Dore J., Marteau P., Seksik P., “Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbita analysis of Crohn disease patients,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 4, pp. 16731–16736, 2008.
[56] A. Sánchez and A. Vázquez, “Bioactive peptides: A review,” *Food Qual. Saf.*, vol. 1, no. 1, pp. 29–46, 2017.
[57] A. Sánchez, A. Vázquez, and L. De Investigación, “Bioactive peptides : A review,” pp. 29–46, 2017.
[58] C. Simango, “POTENTIAL USE OF T R A D I T I O N A L F E R M E N T E D FOODS FOR W E A N I N G IN ZIMBABWE,” vol. 44, no. 7, pp. 1065–1068, 1997.
[59] N. Narayanan, P. K. Roychoudhury, and A. Srivastava, “L (+) lactic acid fermentation and its product polymerization,” *Electron. J. Biotechnol.*, vol. 7, no. 2, 2004.
[60] C. A. O’Bryan, P. G. Crandall, S. C. Ricke, and J. B. Ndahetuye, *Lactic acid bacteria (LAB) as antimicrobials in food products: Types and mechanisms of action*. Elsevier Ltd, 2014.
[61] M. E. Stiles and W. H. Holzapfel, “Lactic acid bacteria of foods and their current taxonomy,” vol. 36, 1997.
[62] K. Khalid, “An overview of lactic acid bacteria,” vol. 1, no. 3, pp.

1–13, 2011.
[63] V. K. Shiby and H. N. Mishra, “Fermented Milks and Milk Products as Functional Foods-A Review,” *Crit. Rev. Food Sci. Nutr.*, vol. 53, no. 5, pp. 482–496, 2013.
[64] A. Kilara, *Regulatory aspects of yogurt*. Elsevier Inc., 2017.
[65] P. K. Singh and N. P. Shah, *Other fermented dairy products: Kefir and koumiss*. Elsevier Inc., 2017.
[66] P. Jäkälä and H. Vapaatalo, “Antihypertensive peptides from milk proteins,” *Pharmaceuticals*, vol. 3, no. 1, pp. 251–272, 2010.
[67] D. Charalampopoulos, R. Wang, S. S. Pandiella, and C. Webb, “<Charalampopoulos_2002_Application-of-cereals-and-cereal-components-in-functional-foods-a-review.pdf>,” vol. 79, pp. 131–141, 2002.
[68] S. Wang, P. Li, J. Yu, P. Guo, and S. Wang, “Multi-scale structures and functional properties of starches from Indica hybrid, Japonica and waxy rice,” *Int. J. Biol. Macromol.*, vol. 102, pp. 136–143, 2017.
[69] S. Lee, J. H. Lee, and H. J. Chung, “Impact of diverse cultivars on molecular and crystalline structures of rice starch for food processing,” *Carbohydr. Polym.*, vol. 169, pp. 33–40, 2017.
[70] M. V. Barone *et al.*, “RET/PTC1 oncogene signaling in PC Cl 3 thyroid cells requires the small GTP-binding protein Rho,” *Oncogene*, vol. 20, no. 48, pp. 6973–6982, 2001.
[71] C. Monaco *et al.*, “The RFG oligomerization domain mediates kinase activation and re-localization of the RET/PTC3 oncoprotein to the plasma membrane,” *Oncogene*, vol. 20, no. 5, pp. 599–608, 2001.
[72] A. Gargiulo *et al.*, “Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement,” *Am. J. Hum. Genet.*, vol. 80, no. 4, pp. 751–758, 2007.
[73] M. Lombardi *et al.*, “Hormone-dependent nuclear export of estradiol receptor and DNA synthesis in breast cancer cells,” *J. Cell Biol.*, vol. 182, no. 2, pp. 327–340, 2008.
[74] E. Zagato *et al.*, “Lactobacillus paracasei CBA L74 Metabolic Products and Fermented Milk for Infant Formula Have Anti-Inflammatory Activity on Dendritic Cells In Vitro and Protective Effects against Colitis and an Enteric Pathogen In Vivo,” vol. 9, no. 2, 2014.
[75] R. Nocerino *et al.*, “Cow’s milk and rice fermented with Lactobacillus paracasei CBA L74 prevent infectious diseases in children: A randomized controlled trial,” *Clin. Nutr.*, vol. 36, no. 1,

pp. 118–125, 2017.
[76] M. Gallo *et al.*, “Effect of pH control during rice fermentation in preventing a gliadin P31-43 entrance in epithelial cells,” *Int. J. Food Sci. Nutr.*, vol. 0, no. 0, pp. 1–9, 2019.
[77] G. K. Gbassi and T. Vandamme, “Probiotic encapsulation technology: From microencapsulation to release into the gut,” *Pharmaceutics*, vol. 4, no. 1, pp. 149–163, 2012.

Appendix I

List of publications:

I. Rice fermentation of *Lactobacillus paracasei* CBA L74.
Gallo M, Nigro F, Passannanti F, Salameh D, Budelli A, et al. (2018). International journal of rice research .IJRR-103. DOI: 10.29011/IJRR-103. 000003.

II. Fermentation of rice flour supernatant using Lactobacillus Paracasei CBA L74.
Salameh D, Nigro F, ColucciCante R, Passannanti F, Gallo M, Budelli A, Marzocchella A, Nigro R. Chemical engineering transactions. Vol 76, 2019.

III. Effect of pH control during rice fermentation in preventing a gliadin P31-43 entrance in epithelial cells.
Marianna Gallo, Federica Nigro, Francesca Passannanti, Merlin Nanayakkara, Giuliana Lania, Francesca Parisi, Dana Salameh, Andrea Budelli, Maria Vittoria Barone & Roberto Nigro (2019): International Journal of Food Sciences and Nutrition, DOI: 10.1080/09637486.2019.1599827

IV. Lactic fermentation of cereal flour: feasibility tests in rice, oat and wheat. GALLO, Marianna et al. Applied Food Biotechnology, [S.l.], v. 6, n. 3, p. 165-172, june 2019. ISSN 2423-4214. DOI: https://doi.org/10.22037/afb.v6i3.24299.

V. Effects of the glucose addition during lactic fermentation of rice, oat and wheat flours.
Marianna Gallo, Francesca Passannanti, Rosa ColucciCante, Federica Nigro, Dana Salameh, Paola Schiattarella, Concetta Schioppa, Andrea Budelli and Roberto Nigro.
Applied Food Biotechnology, [S.l.], v. 1, n. 1, p. 21-30, dec. 2019. ISSN 2423-4214. https://doi.org/10.22037/afb.v1i1.26975.

VI. Biotechnological production of natural sweeteners and preservatives on tomato paste.
Marianna Gallo, Francesca Passannanti, Elena D'Apolito, Federica Nigro, Dana Salameh, Roberto Nigro. Journal of food process engineering. 4 Dec. 2019. https://doi.org/10.1111/jfpe.13327.

List of abbreviations

L. para	Lactobacillus paracasei
RP-HPLC	Reversed phase-High pressure liquid chromatography
GC	Gas chromatography
h	Hours
mg	Milligrams
mL	Millilitre
CFU	Colony forming unit
rpm	Round per minute
T	Sampling time
LAB	Lactic acid bacteria
MRS	De Man, Rogosa and Sharpe agar
PID	Proportional integral derivative
DMEM	Dulbecco's modified eagle medium
KDa	Kilo Dalton
RT	Room temperature
Maldi TOF	Matrix assisted laser desorption/ionization time of flight
M	Molar
S	Seconds
U	Unit
MW	Molecular weight
TFA	Trifluoroacetic acid
CAN	Acetonitrile
FBS	Foetal bovine serum
WCB	Working Cell Bank
MCB	Master Cell Bank
m/z	Mass to charge ratio
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
CO2	Carbon dioxide
GRAS	Generally regarded as safe
PFR	Plug flow reactor
-OH	Hydroxide
-NH2	Azanide
COO-	Carboxylate group
NH3	Ammonia
ID	Internal diameter
UHT	Ultra high temperature

Kcal	Kilo calorie
H2O	Water
G	Gram
DV	Dailey value
Gel	Gelisate
Mc	MacConkey
mM	Milli Molar
Kg	Kilogram
PLC	Programmable logic controllers
w/v	Weight to volume
V	Volume
Wp	Lactic acid productivity
Wx	Biomass productivity
Ks	Semi-saturation constant
Umax	Maximum specific growth rate
S	Substrate
Y	Yield
K	Growth constant
Y (S/X)	Biomass yield
Y (P/X)	Product yield
N	Number of generation
°C	Celsius grade
Cm	Centimetre
BAF	Animal free broth
NaOH	Sodium hydroxide
Nm	Nanometre
A	Angstrom
Mm	Millimetre
Da	Dalton
µL	Microliter
DTT	Dithiotheritol
IAA	Indole -3-acetic acid
CAN	Acetonitrile
EtOH	Ethanol
HCCA	α- cyano-4-hydroxycinnamic acid matrix
D	Diameter
PBS	Phosphate buffered saline
GOS	Glactooligosaccarides
FOS	Fructooligosaccharides
TOS	Transgalactooligosaccarides

TNF-α	Tumor necrosis factor- α
CRP	C reactive protein

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Foreign experience

The foreign experience was from the 1st of may 2019 until the 31st of July 2019. It was done at the Kraft Heinz innovation centre (57) in Nijmegen, The Netherlands, under the supervision of Dr. Andrea Budelli.

Address: Nieuwe Dukenburgseweg 19

6534 AD Nijmegen

Schools, workshops, courses and seminars during PhD

Seminars:

1. How smart a cell can be? Do transport phenomena play a role in dynamic evolution of cell systems? Prof. Caserta. 8-02-2017, Department of chemical science
2. From the farm to the gut: mechanisms of bacterial adaptation. Prof. Sacha Lucchini. 22-02-2017, Department of biology

3. Il processo di sviluppo di un prodotto alimentare, part 1. Dr. Andrea Budelli. 8-05-2017, DICMAPI.
4. Il processo di sviluppo di un prodotto alimentare, part 2. Dr. Andrea Budelli. 18-05-2017, DICMAPI.
5. Il breif del prodotto. Dr. Andrea Budelli. 22-05-2017, DICMAPI.
6. Comprensione del ruolo di p63 durante lo sviluppo embrionale. Prof. Luisa Guerrini. 23-05-2017, Department of biology.
7. Fungal membrane bioreactor and photocatalytic Processes in industrial wastewater. Prof. Ece Ummu Deveci. 24-05-2017, DICMAPI.
8. Role of p63 and p73 in cutaneous squamous cell carcinoma. Dott. Dario Antonini. 13-06-2017, Department of biology.
9. The Bioliq-BTL- process line: from basic research to pilot plant operation. Prof. Dr-Ing Thomas Kolb. 26-07-2017, DICMAPI.
10. Ring closing metathesis for the formation of 9 membered benzo-fused rings. Prof.Ivan Robert Green. 21-09-2017, Department of chemical science.
11. Dynamic control: mathematical challenges and applications. Prof. Enrique Zuazua. 17-01-2018, DICMAPI.
12. Biotecnological applications of bioactive peptides and peptidomimetics: peptides and peptidomimetics for cosmetic applications and food industry, Nanotechnology: peptides as biological semiconductors. Dr.Angela Arciello. 18/19-05-2017, Department of chemical sciences.
13. Enzymes in food biotechnology: enzymes as processing additives, enzymes as processing aids. Dr. Concetta Valeria L.Giosafatto. 25/26-05-2017, Department of chemical sciences.

14. Protein structure visualization,analysis and modelling. Prof. Eugenio Notomista. 5-05-2017, 9-05-2017,9-06-2017, Department of biology.
15. Microbial cell factories in industrial biotechnology, Design and optimization of microbial cell factories for industrial bioprocess. Dr. Cinzia Pezzella. 22/23-06-2017, Department of chemical sciences.
16. Anti adhesive effect of oligosaccharides microflora using cell culture model. Dr.Mohammad Altamini. 7-07-2017, Department of chemical sciences.
17. Immuno modulatory antimicrobial peptides: biology and applications. Prof. Henk Peter Haagsmann. 15-02-2018, Department of biology.
18. Nanotecnologie ed imaging fotoacustico: nuovi approcci per l'imaging molecolare. Dr. Luca Menichetti. 23-02-2018, DICMPI.
19. Regulation of X chromosome inactivation by Xist long non-coding RNA. Prof. Andrea Cerase. 6-03-2018, Department of biology.
20. Advanced materials by external controlled ATRP. Prof. Krzysztof Matyjaszewaki. 16-06-2018, DICMAPI.
21. The potential of electrospun nsnocomposite nanofibers in chemical sensor for gas pollutants. Prof. Antonella Macagnano. 4-04-2018, DICMAPI.
22. Digital manufacturing of microfluidic devices. Prof. Dr. Albert Folch. 10-05-2018, DICMAPI.

23. En route to synthetic biology: discussion from several examples. Prof. Thierry Tron. 22-06-2018, Department of chemical sciences.
24. Bacterial spore as Nanoparticle display platform, New generation of Heavy metal biosensor, Hydrogen production by the thermophilic bacterium T.naepolitana. Dr. Isticato, Dr. Varriale, Dr.Fontana. 11-10-2018, Department of chemical sciences.
25. Genome editing. Dr. Saccone, Prof. Falco, Prof. Missero. 15/16-10/2018. Departemnt of chemical sciences.
26. Transient techniques in the investigation of catalytic three-phase processes. Prof. Tapio Salmi. 12-11-2018, DICMAPI.
27. Bio-stimulation of plant endogenous defences. Dr. Mariangela Coppola. 13-11-2018, Department of agriculture.

Schools:

Spring school on transferable skills. 14-15/ 05/ 2018. Department of pharmacy. Dr. Pasquale Maffia, Prof. Jim Brewer, Fiona Stubbs, Prof. Valeria Costantino.

Congresses

1. Itpa: feeding the proteopme. 5-7/ 9/ 2018. University of Insubria- Como, Italy.
2. Eff 2nd conference: Engineering future food. 26-29/ 05/ 2019. Bologna, Italy.
3. 2nd industrial biotechnology congress: BioID&A; Biotechnology Identity and applications. 28/10/2019. University of 'Federico II', Naples, Italy.

Oral presentation:

Oral presentation of “Fermentation of rice flour supernatant using Lactobacillus Paracasei CBA L74” (Salameh D, Nigro F, ColucciCante R, Passannanti F, Gallo M, Budelli A, Marzocchella A, Nigro R. Chemical engineering transactions. Vol 76, 2019) - EFF 2nd conference, Bologna, May 26-29 2019.

Appendix II

A.1 Plate count is a method used to enumerate microorganisms present in a sample, it is based on the use of general or semi selective media formulated with respect to the demands of the specific microbial groups. Plate count results reflect the number of colonies that can emerge under the given physical and chemical conditions (atmosphere, temperature, pH, available nutrients, presence of growth inhibitory compounds).
Direct plate counting is a method used to count the number of viable cells in a sample, in order to do so samples must be diluted first otherwise the growth on the Petri dish will be so dense it would be impossible to count. Once the cells have been diluted they are incubated on an agar medium until colonies are formed.
There are two methods to direct plate counting: spread plate method and pour plate method, both these techniques were used to evaluate the bacterial growth:
The spread plate method is a method employed to plate a liquid sample for the purpose of isolating or counting the bacteria present in that sample. A perfect spread plate technique results in visible and isolated colonies. Usually the sample is diluted using the serial dilution method (A.5), then a volume of 0.1 ml of the diluted sample is spread across an agar Petri dish. After incubation the colonies are formed and it is possible to count them.
Pour plate method on the other hand, requires that the sample is pipetted in a sterile Petri dish and liquid agar is poured onto the sample and mixed with it. Using this method bigger volumes of diluted samples could be used.

A.2 Matrix assisted laser desorption/ionization- Time of Flight:
The MALDI is an ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation.
This method has been applied to the analysis of biomolecules (DNA, proteins, peptides and sugars) and large molecules (polymers and other macromolecules) that could be fragmented when ionized by more conventional ionization methods. Figure (55) shows the ionization of analytes by MALDI.

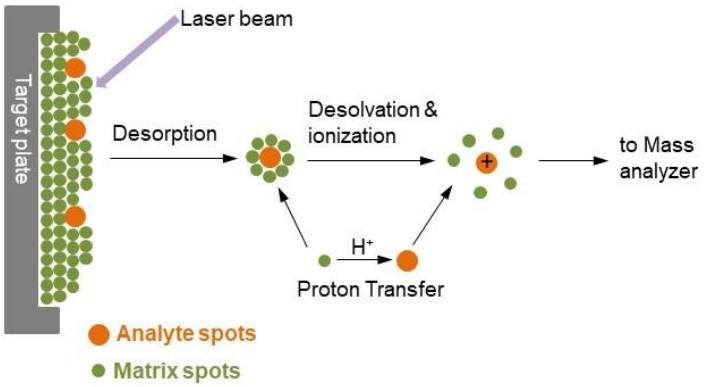

Figure 55: Ionization of analytes by MALDI

The MALDI is a three step process:

1. Sample is mixed with a suitable material and applied to a metal plate that has spots for several different samples to be applied;
2. A pulsed laser irradiates the sample, triggering ablation and desorption of the sample and matrix material;
3. The analyte molecules are ionized by being protonated or deprotonated in the hot plume of ablated gases and they can be accelerated into whichever mass spectrometer is used to analyze them.

Lasers of both ultraviolet and infrared wavelengths are in use.

Time of flight (TOF) is a mass spectrometer most widely used in combination with MALDI. It has a large mass range. In this case, the pulsed laser takes individual ‘shots’ instead of working in continuous operation. MALDI-TOF instruments often are equipped with a reflectron, that reflects ions using an electric field. This increases the ion flight path, thereby, increasing time of flight between ions of different m/z (mass to charge ratio) and increasing the resolution. The lighter ions will arrive earlier to the detector than the heavier ones.

Matrices used to perform the analysis differ depending on the type of molecule to analyze. Table (26) reports some examples of matrices and their application:

Table 26: Examples of matrices used in MALDI analysis and their applications

Compound	Acronym	Application to
Nicotinic acid	NA	Peptides, proteins
Picolinic acid	PA	Oligonucleotides, DNA
6-AZA- 2 thiothymine	ATT	Oligonucleotides, DNA
α-Cyano-4-hydroxycinnamic acid	HCCA	Peptides, smaller proteins
2-(4-Hydroxyphenylazo) benzoic acid	HABA	Peptides, proteins, glycoproteins
5-Chloro-2-mecaptobenzothiazole	CMBT	Glycopeptides, phospohopeptides and proteins

A.3 pH meter: is a scientific instrument that measures the hydrogen-ion activity in water-based solutions, indicating its acidity or alkalinity expressed as pH. The pH meter measures the difference in electrical potential between a pH electrode and a reference electrode. A typical pH meter uses a probe that measures and displays the pH value.
The probe used is In Pro 3100i Mettler Toledo probe, which is a glass probe with a diameter of 12 cm and a length of 15cm.

A.4 Freeze drying: is a low temperature dehydration process which involves freezing the product, lowering pressure then removing the ice by sublimation. Freeze-drying results in high quality products because of the low temperature used in processing. The apparatus used is a bench freeze-drier Alpha 1-2 LD-plus. This method has the advantage of causing less damage to the substances compared to other dehydration methods which use higher temperatures. The characteristics of the dried material such as smell and flavour aren't altered by this process. The main disadvantages of this method is the high cost and low quantities processed with each cycle.
The process is divided into three steps:

- Pre-treatment of the sample: which consists in stabilizing the sample before freezing it. The stabilization consisted in lowering the microbial charge through heat treatment (90°C/ 1 min);
- Freezing: samples should be frozen for at least 24h at -26° C;
- Lyophilization: this step consists in
    - Warm up of the vacuum for 15 min;
    - Primary or main drying for 24 h during which the pressure is lowered to the range of millibars for the ice to sublime. In this initial phase about 95% of water in the material is sublimated.

- Secondary or final drying for 24 h which aims to remove unfrozen water molecules since the ice was removed in the previous phase. This part of the freeze drying is governed by the material's adsorption isotherms. Temperature is raised higher than that in the primary phase and can even be above 0°C to break the physio-chemical interactions that were formed between water molecules and the frozen material. The pressure is usually lowered in this stage to encourage desorption.

Sublimation is the transition of a substance directly from the solid to the gas phase without passing through the intermediate liquid phase. It is an endothermic process that occurs at temperatures and pressures below a substance's triple point in its phase diagram, which corresponds to the lowest pressure at which a substance can exist as a liquid. In freeze-drying the material to be dehydrated is frozen and its water is allowed to sublime under reduced pressure or vacuum. (Figure 56)

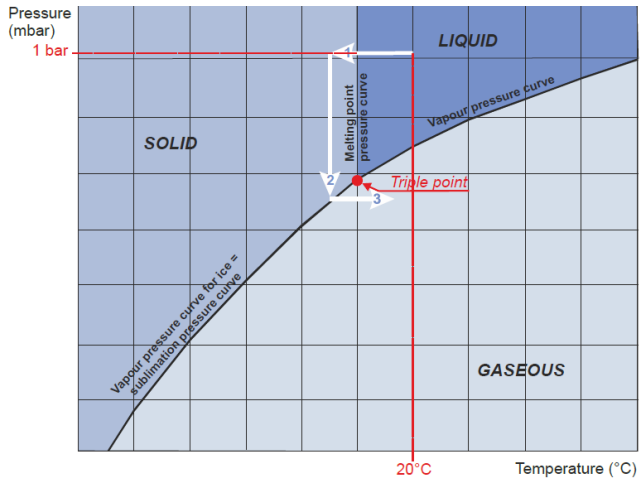

Figure 56: freeze-drying phases

A.5 Serial dilution: is the stepwise dilution of a substance in solution. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in logarithmic fashion.

A.6 Reversed Phase -High Performance Liquid Chromatography

HPLC is a common technique which is able to separate and quantify compounds in a mixture, usually using operational pressures significantly higher than ordinary liquid chromatography. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. The separation of the molecules depends on different parameters:

1. Internal diameter (ID): of the column used influences the detection sensitivity and separation selectivity in gradient elution.
2. Particle size: particles are attached to the silica in the column. Small particles generally provide more surface area and better separation;
3. Pore size: many stationary phases are porous to provide higher surface area;
4. Pump pressure: determines the volumetric flow rate;
5. Detectors: they are divided into two main categories; universal that measure a property by detecting differences between mobile and stationary phase; while selective detectors measure a solute property by responding to the physical or chemical property of the solute.

Reversed phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase.

RP-HPLC operates on the principle of hydrophobic interactions; which originate from high symmetry in the dipolar water structure and play the most important role in all processes in life science. RP-HPLC allows the measurement of those interactive forces. The binding of the analyte to the stationary phase is proportional to contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a large hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) is retained longer because it is non-interacting with the water structure. On the other hand, analytes with higher polar surface area (conferred by the presence of polar groups, such as –OH, $-NH_2$, COO^- or NH_3^+ in their structure) are less retained as they are better integrated into water.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly, organic compounds with single C-C bonds elute later than those with C=C or C-C triple bond, as the double or triple bond is shorter than a single C-C bond.

Another important factor is the mobile phase's pH; its values can change the hydrophobic character of the analyte.

A.7 Gel electrophoresis: is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments based on their size and charge. It involves a running current through a gel

containing molecules of interest. Gels' agarose is made from natural polysaccharide polymers extracted from seaweed. They have different pore size.

A.8. Proportional Integral Derivative (PID): PID controller is an instrument used in industrial control applications to regulate temperature, flow, speed and other process variables. PID controllers use a control loop feedback mechanism to control process variables and are the most accurate and stable controllers.

A.9 Spray drying: is a method of producing a dry powder from liquid or slurry by rapidly drying with hot gas. It is usually used by industries to produce foods and pharmaceuticals. The method uses heated air as drying medium. A type of spray nozzle or atomizer is used to disperse the liquid or slurry into a controlled drop size spray.[77]
This process is divided into five steps:

- Feed preparation: the suspension or paste should be a homogenous pumpable feed free of impurities;
- Atomization: in this step the dry rate is controlled and therefore the dryer size
- Drying: a constant rate phase ensures moisture evaporates rapidly from the surface of the particle. This is followed by falling rate period where the drying is controlled by diffusion of water to the surface of the particle.
- Separation of powder from moist gas: to be carried in an economical and pollutant-free manner. Fine particles are generally removed with cyclones, bag filters, precipitators or scrubbers.
- Cooling

A.10 Master cell bank (MBC): is an aliquot of single pool of cells that generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The MBC is used to derive all working cell banks (WBC) which is prepared from aliquots of a homogenous suspension of cells obtained from culturing the MCB under defined culture conditions.

A.11 YSI 2900 Biochemistry analyser: is an instrument that analyses the concentration of a substrate in a product by using enzyme sensor technology.
YSI's enzyme sensor technology employs one or more enzyme catalysed reactions to ultimately produce hydrogen peroxide (H_2O_2) as shown in Figure (57).

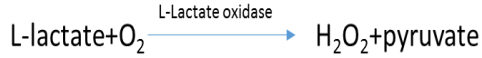

Figure 57: YSI work principle through oxidization of L-lactate

Each enzyme probe is fitted with a three-layer membrane containing the immobilized enzyme. The face of the probe, covered by the membrane, is situated in a buffer-filled sample module into which a sample is injected. Some of the substrate diffuses through the membrane. When it contacts the immobilized oxidase enzyme, it is rapidly oxidised producing hydrogen peroxide; which in turn oxidized at the platinum anode produces electrons. A dynamic equilibrium is achieved when the rate of H_2O_2 production and the rate at which H_2O_2 leaves the immobilized enzyme layer are constant and it is indicated by a steady state response. The electron flow is linearly proportional to the steady state H_2O_2 concentration and therefore to the concentration of the substrate.

Aknowledgments

I won't pretend this isn't the best part about writing this entire thesis!

I'd like to start by thanking my professors for their support and guidance throughout these years, especially Prof. Marzocchella and Prof. Nigro.

To everyone in food engineering lab; Marianna, Francesca, Rosa, Marica, Paola, Tina, Annalisa, Valentina, Fabio, Carlo, Giulia, Vincenzo and Francesco, thanks not only for the laughs and the great memories, but most importantly, for teaching me Napoletan. I couldn't have gone through this without Federica, the best partner in crime anyone could have asked for, for being sweet, generous, kind, independent yet so fiercely feminine, I learned a lot from you.

Although we are 3387.4 Km apart, a great thank you goes to Nuran & Georgette; I could have never asked for better friends. A special thank you goes to Hanna, one rarely finds a friend like you.

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All that I have achieved in my life I owe to my family…

To Aloon the best mother EVER! For all the sacrifice, for every tear, for every kiss and hug…thank you for showing me how to love.

To Abu el3ezz, through example, perseverance and hard work, thank you for showing me how to 'get that DAMN monkey'! ... I could stop here, but then the other three most important people in my life would get offended, so here we go… (in order of size so no one would get offended), to Zuzu, Nunu & Dede, for being kind, wise, supportive, funny, sarcastic, similar and yet so unique…my life would have been so boring without the three of you, thank you for teaching me how to be a better me. Thank you as well, to the -not so new- members of this family, Chiara & Alessio, having an extra brother and sister is always amazing!!

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I would end here, but I think my future self would be offended…I'd like to thank myself, for not giving up... We did it!

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Rice Fermentation by Lactobacillus Paracasei CBA L74

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Abstract

In recent decades, the increase in economic wellbeing and in nutrition awareness have increased the attention of consumers towards the high-quality food. In particular, consumers pay attention to the effects of food on physiological functions, and to the connections between food components and the prevention of some pathologies. Potential solutions to the consumer interests are the functional foods. A wide spectrum of probiotic foods may be produced by bacterial fermentation of milk.

This contribution reports preliminary results regarding the development of a functional semi-finished food by fermentation of *Lactobacillus Paracasei* CBA L74 using rice flour as substrate. The process was investigated by tests carried out at a laboratory scale. Batch fermentations were carried out in a 1.5 L fermenter equipped with a thermostatic system. The medium was a suspension of rice flour and water.

The fermentation process was characterized in terms of pH, microbial population, primary (lactic acid) and secondary acid (butyric and acetic acids) concentration. The maximum bacterial charge of $3*10^8$ CFU/ml achieved during the process and the high production of lactic acid of 222,4 mg/L suggested that the conditions implemented throughout the fermentation process could be appropriate for the production of a rice flour based functional food.

Keywords: Batch Reactor; Fermentation; Functional Foods; Lactobacillus Paracasei; Mixing System

Introduction

From last scientific evidence, functional foods are acquiring more attention due to their beneficial effect on human health and prevention of diseases. The concept of functional foods includes foods or food ingredients that exert a beneficial effect on host health and/or reduce the risk of chronic diseases beyond basic nutritional functions [1]. Probiotic foods contain a single or mixed culture of microorganisms that affect beneficially the consumer's health by improving their intestinal microbial balance [2].

Fermentation is one of the oldest and most economical methods of producing and preserving food [3]. In addition, fermentation provides a natural way to destroy undesirable components, to enhance the nutritive value and appearance of the food [4]. An appropriate selection of the strain is necessary to efficiently control the different types of the metabolic end products [5-7].

Lactic acid fermentation is one of the most important food processing technologies to produce probiotic foods. Today most probiotics available on the market are dairy based providing several types of fermented milks and cheeses [8]. Several studies (conducted *in vivo* and *in vitro*) reported in the literature point out a potential positive effect of the fermented dairy based foods on the human health [9-11].

The food industry is directing new product development towards the area of functional foods and cereals could offer an alternative for the production of this type of foods. Cereals are widely harvested worldwide and contribute to over 60% of the world food production [12] providing dietary fibre, proteins, energy, minerals, and vitamins required for human health. Although they

are deficient in some basic components such as essential amino acids, fermentation may be a simple and economic way to improve their nutritional value. Furthermore, the non-digestible components in the cereal matrix may act as prebiotics and propose the cereal as a good substrate for the growth of probiotic strains [12,13].

There are some proofs that cereals may be a healthier option to produce new non-dairy probiotic foods. Indeed, they can overcome some of the disadvantages associated with fermented dairy products like lactose intolerance, allergy and the contribution to the increase of the cholesterol concentration in the blood stream [14].

The possible applications of cereals or cereal constituents in functional food formulations could be summarised: as fermentable substrates for growth of probiotic microorganisms, especially Lactobacilli and Bifidobacteria, as dietary fibre promoting several beneficial physiological effects, as prebiotics due to their content of specific non-digestible carbohydrates, as encapsulation materials for probiotic in order to enhance their stability. The possibility of fermenting a solid cereal based matrix has been already investigated, in particular in a study using water based pudding with 75% rice and 25% maize, with and without the addition of Litesse™ -a fibre with prebiotic properties. The growth and metabolism of four strains of lactobacillus was evaluated. Starting with a bacterial charge of 7 log CFU/g, the final charge of bacteria reached was 8 log CFU/g with a lactic acid production of 560-640 mg/Kg. The fermentation was carried out in glass jars for 12h with no agitation. Growth and metabolism of the selected strains of probiotic bacteria were not affected by the addition of Litesse™ [15].

The present paper reports on a recent investigation regarding the fermentation process using cereal flour as a substrate to produce ingredients of functional foods. Rice flour was used as substrate for a culture fermentation of *Lactobacillus Paracasei* CBA L74. The fermentation was characterized in terms of pH, bacterial growth, lactic acid and secondary acids production. Also kinetic parameters, such as productivity, biomass and product yield were evaluated.

Materials and Methods

Strain and Substrate

The microorganism was *L. paracasei* CBA L74 (Heinz Italia SpA, Latina, Italy), a gram positive homofermentative, facultative anaerobic bacteria. International Depositary Accession Number was LMG P-24778. The bacterial stock was stored at -26° C. The lyophilized starter culture with a potential bacterial charge of 10^{11} CFU/ml was revitalized in MRS broth by incubation at 37°C for 24h. The fermentation substrate, rice flour, was provided by Heinz Italia s.p.a. and stored at 4°C.

Apparatus

Fermentation tests were carried out in a batch reactor of 1.5-L. The experimental laboratory system consisted essentially of four components: vessel, mixing system, thermal conditioning system and temperature measurement system. The vessel was cylindrical Pyrex (20 cm high, 10 cm ID) equipped with an external jacket that allowed the circulation of a service fluid necessary to maintain the entire apparatus at a constant temperature. The fluid was thermo regulated through a thermostatically-controlled water bath.

The mixing system was a stainless-steel impeller equipped with an inclined blade turbine and a Rushton turbine that were used to provide axial and radial flow to guarantee homogeneity of the matrix and avoid gradient temperature and microorganism concentration. The impeller was linked to a motor that allowed to adjust the stirring speed (in this case it was 180 rpm chosen for a substrate viscosity of about 1.15 [Pa. s]). The impeller had an input connected to a probe, useful for measuring automatically the temperature simultaneously to the process' progress.

Operating Conditions and Procedure

The working volume was set at 1-L. Substrate composition was 85% of water, 15% of rice flour. The fermentation tests were carried out at 37°C and the total time of process was approximately 24 h. Before each fermentation, the rice was dry heated at 121°C for 90 minutes whereas each component of the system was sterilized by a standard autoclave cycle (121°C for 20 minutes). Moreover, the rice suspension was tyndallized, in order to kill vegetative forms of bacteria. Before the start of fermentation, the suspension in the reactor was subjected to two thermal cycles each formed by subsequent heating (at 70°C for 30 minutes) and cooling (at 37°C for 30 minutes).

This heat treatment caused the gelatinization of the starch in the suspension hence the use of the impeller previously described to guarantee homogeneity of suspension. Once the system was stable at 37°C, the cells under active growth state were inoculated in the fermenter. During the 24h of the process, withdrawals were done to evaluate the bacterial growth rate, pH and the production of primary and secondary acids.

Analytical Methods

The viscosity of the suspension, after the tyndallization phase was determined vs shear rate using a stress-controlled rheometer (SR200, Rheometric Scientific) equipped with parallel plates geometry of 40mm diameter. The temperature was kept constant by using a Peltier apparatus, which ensures an accuracy of ±1°C. The gap of the measurements was kept fixed to1mm. Rheological tests were carried out at 30°C. For each sample, the same test was performed three times, by loading a fresh sample each time. The

presence of micro-agglomerates in the samples did not allow the use of the cone-plate geometry that would have been preferred to parallel plates in non-linear regime.

To analyse cell growth, production of organic acids and pH values, samples were withdrawn aseptically from the bioreactor at different times up to 24h. Samples of the fermented matrix were transferred on Petri Plate to monitor the growth of lactobacilli on MRS (De Man, Rogosa and Sharpe, oxoid) agar plates using the spread plate method. Specific agar plates such as Mac Conkey (Oxoid) and PCA **(**Oxoid) were used to control contaminant presence. All plates were incubated for 24h at 37°C.

Lactic acid concentration was measured by using an HPLC (Agilent technologies 1100 with Visible/UV detector). Agilent Zorbax C18 column (4,6mm x 150 mm and a pore size of 80A) was used. The flow rate was set at 0.8mL/min; the mobile phase was ammonium phosphate with a pH of 2.7 with detection at 218 nm. Butyric and acetic acids concentration was carried out by gas chromatography (Agilent Technologies 6890). Capillary Poraplot Q column (25m x 0.32mm) was used. The flow rate was 200 ml\min. The mobile phase was helium gas.

The pH was measured in culture samples using a bench-top pH-meter mod. Expandable IonAnalyzer EA920-Orion Research. The substrate concentration (starch) consumed by lactobacillus during fermentation was measured by an enzymatic kit- Analytical method according to AOAC method 996.11, AATC method 76.13 and ICC standard method No.168.

1.1. Kinetic Parameters

In addition, the process was characterised in terms of: productivity, biomass and product yield. The biomass production throughout the fermentation process (*P* [mg/h], Productivity of Fermentation Process, PFP) was calculated according to the Eq. (1):

$$P = \frac{v\,tot * x_{biomass}}{t_{fermentation} + t_{dead}} \qquad (1)$$

Where v_{tot} [L] is the total volume of suspension, $x_{biomass}\,[mg/L]$ is the biomass produced during process; to switch from CFU/ml to mg/ml the conversion factor was calculated experimentally after 24 h of incubation, *L. Paracasei* CBA L74's growth was evaluated through spread plate method and through spectrophotometer examinations to verify if the final bacterial charge was comparable in both methods, after which the culture was centrifuged and the pellet obtained was weighed and the ratio between the weight of the latter and the number of CFU/ml read on the plates was $1*10^{-9}$. $t_{fermentation}$ [h] the time of process, and $t_{dead}\,[h]$ the dead time of the fermentation process.

The amount of biomass produced to the amount of substrate consumed (Y_{sx}, Biomass Yield, BY) was calculated according to Eq (2):

$$Y_{sx} = \frac{\Delta X}{\Delta S} = \frac{X_{fin} - X_0}{S_0 - S_{fin}} \qquad (2)$$

Where $X_0\,[mg/L]$ is the initial concentration of biomass, X_{fin} [mg/L] is the final concentration of biomass, $S_0\ [mg/ml]$ is the initial concentration of substrate, S_{fin} [mg/L] is the final concentration of substrate. The amount of lactic acid produced respect to the amount of substrate consumed (Y_{sp}, Product Yield in Lactic Acid, LAPY) was calculated according to Eq (3):

$$Y_{sp} = \frac{\Delta P}{\Delta S} = \frac{P_{fin} - P_0}{S_0 - S_{fin}} \qquad (3)$$

Where P_0 [mg/L] is the initial concentration of lactic acid, $P_{fin}\ P_{fin}$ [mg/L] is the final concentration of lactic acid, $S_0\,S_0$ [mg/L] is the initial concentration of substrate, $S_{fin}S_{fin}$ [mg/L] is the final concentration of substrate.

Result

Considering the difficulties of working with high viscosity, firstly, we had to study the fluid dynamics parameters to guarantee the best condition of homogeneity. The viscosity of suspension analysed ranged from 1÷3 [Pa. s] for a range of 60÷400 [rpm], with a shear rate ranging from $125s^{-1}$ to $837s^{-1}$. The innovation of our work was the improvement of the process thanks to the use of a closed batch reactor equipped with an adequate mixing system.

In relation to the high values of viscosity measured, in fact, two kind of turbines have been selected: an inclined blade turbine and a Rushton turbine in order to obtain both axial and radial movement. Secondly, as indicators of a good performance of the fermentation, high values of CFU counts and high production of lactic acid were taken into account.

The trend of the growth curve is reported in (Figure 1) whereas lactic and secondary acids production is represented in (Figure 2). All values reported in growth and organic acids production curves, are the result of a triplicate mean value. As shown in (Figure 1), starting with an initial bacterial charge of $3,7*10^6$ CFU/ml, the maximum growth is achieved at 17h with a bacterial charge of $3*10^8$ CFU/ml; at the end of the fermentation $2*10^8$ CFU/ml was reached. This data indicated that the exponential phase start at 4h and ends at 17h.

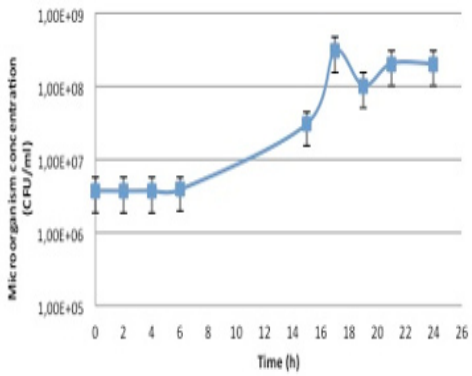

Figure 1: Growth curve.

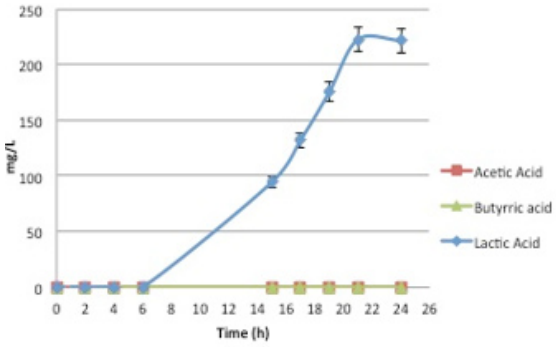

Figure 2: Organic acids.

The highest concentration of lactic acid achieved, at 24h, was 221.6 mg/L, as shown in (Figure 2) corresponding to the lowest value of pH registered at 3.67 as shown in (Figure 3). Moreover, the presence of other acids (butyric and acetic) was not detected, indicating that the fermentation process was conducted in sterile conditions and that *Lactobacillus paracasei CBA L74* is an obligatory homo-fermentative producing high levels of lactic acid.

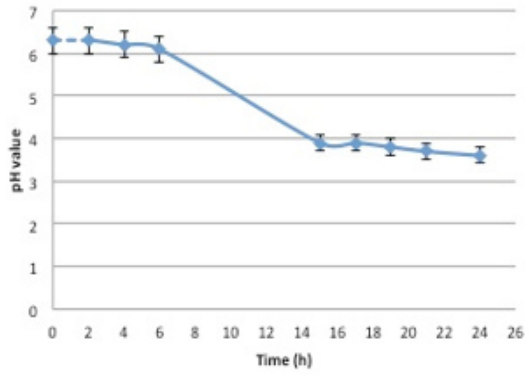

Figure 3: pH value.

The concentration of substrate was studied too, in particular, starch concentration was analysed at different times during fermentation process. From enzymatic analysis, it is possible to have an initial starch concentration of about 67 mg/ml, this concentration decreases during fermentation reaching a quantity of 19 mg/ml at the end of the fermentation.

In these conditions, *L. paracasei* CBA-L74 was able to grow in the rice flour- water suspension in batch fermentation. The productivity of the fermentation was calculated using the Eq. (1). The process achieved a productivity PFP of 5.76 mg/h. The biomass and product yield were calculated using Eq (2) and (3) respectively. The process achieved a biomass yield, BY of 0.026, and a product yield, LAPY of 0.177.

This particular lactobacillus has shown a good performance in gelatinized substrates with final bacterial concentration of 10^8 which is higher than that requested by probiotic standards. In fact, based on the literature available, a minimum level of more than 10^6 viable probiotic bacteria per millilitre or gram of food product is accepted [16].

Similar results to ours have been collected by [17] that studied the performance of L. Helveticus in a multi cereal beverage where the optimal substrate consisted of 50% malt, 25% rice and 25% maize. In its study, Jing Ai added, before the start of fermentation, α-amylase and glucoamilasi, to avoid the gelatinization of the starch; then the matrix was filtered obtaining a liquid matrix.

The latter was then autoclaved at 121°C 15 minutes. The fermentation was carried out 37°C for 24 hours with no pH control reaching a cell concentration of 8.43 log CFU/ml and a production of 1.46 mg/ml of lactic acid at the end of the fermentation.

Discussion

This process demonstrated the efficiency of the fermentation at a laboratory scale. As seen we were able to perform a lactic fermentation, obtaining a product with bacterial charge sufficient to produce a potential functional food. The innovation of our work was the improvement of the process thanks to the use of a closed batch reactor equipped with a mixing system adequate to viscosity of product, by which we obtained homogeneity of the conditions. The fermentation was performed on a rice suspension, a rich source of vitamins, prebiotics, minerals but with a high viscosity.

The product of fermentation process is characterized by a starch concentration of 19 mg/ml with a substrate consumption of about 48 mg/ml, a cell concentration of 8.43 log CFU/ml and a production of 1.46 mg/ml of lactic acid at the end of the fermentation. In conclusion, with a starch consumption of about 70% it is possible to have an increase of cell concentration of two log CFU/ml associated with a lactic acid production, obtaining a product with probiotic potential. Future prospects will be the

improvement of the process and the verification of efficiency of this process in pilot and industrial scale.

References

1. Huggett AC, Schliter B (1996) Research needs for establishing the safety of functional foods. Nutrition Review 54: 143-148.
2. Fuller R (1989) Probiotics in man and animals. Journal of applied Bacteriology 66: 365-378.
3. Chavan JK, Kadam SS (1989) Nutritional improvement of cereals by fermentations. Crit Rev Food Sci Nutr 28: 349-400.
4. Simango C (1997) Potential use of traditional fermented foods for weaning in Zimbabwe. Journal of Social Science and medicine 44: 1065-1068.
5. Lönner C, Preve-Åkesson K (1988) Acidification properties of lactic acid bacteria in rye sour doughs Food microbiology. Elsevier 5: 43-58.
6. Damiani P, Gobbetti M, Cossignani L, Corsetti A, Simonetti MS, et al. (1996) The sourdough microflora. Characterization of hetero and homofermentative lactic acid bacteria, yeast and their interaction on the basis of the volatile compounds produced. Food science Technology 29: 63-70.
7. Hansen A, Lund B, Lewis MJ (1989) Flour production and acidification of sourdough in relation to the starter culture and fermentation temperature. Lebensm Wiss u Technol 22: 145-149.
8. Gomes AMP, Malcata XF (1999) Bifidobacterium ssp. and Lactobacillus acidophilus: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends in Food Science and Technology 10: 139-157.
9. Nocerino R, Paparo L, Terrin G, Pezzella V, Amoroso A, et al. (2015) Cow's milk and rice fermented with Lactobacillus paracasei CBA L74 prevent infectious diseases in children: A randomized controlled trial. Elsevier. Clinical Nutrition 36: 118-125.
10. Sarno M, Lania G, Cuomo M, Nigro F, Passannanti F, et al. (2014) Lactobacillus paracasei CBA L74 interferes with gliadin peptides entrance in Caco-2 cells. International Journal of Food Science and Nutrition 65: 953-959.
11. Zagato E, Mileti E, Massimiliano L, Fasano F, Budelli A, et al. (2014) Lactobacillus paracasei CBA L74 metabolic products and fermented milk for infant formula have anti-inflammatory activity on dendritic cells *in vitro* and protective effects against colitis and an enteric pathogen *in vivo*. Plos one 9 (2) e87615.
12. Charalampopoulos D, Wang R, Pandiella SS, Webb C (2002) Application of cereals and cereal components in functional foods: A review. International Journal of Food Microbiology 79: 131-141.
13. Charalampopoulos D, Pandiella SS, Webb C (2002) Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. Journal of Applied Microbiology 92: 851-859.
14. Prado FC, Parada JL, Pandey A, Soccol CR (2008) Trends in non-dairy probiotic beverages. Elsevier. Food Research International 41: 111-123.
15. Helland MH, Wicklund T, Narvuhus JA (2004) Growth and metabolism of selected strains of probiotic bacteria in milk-and water-based cereal puddings. International dairy journal 14: 957-965.
16. Lacroix C, Yildrim S (2007) Fermentation technologies for the production of probiotics with high viability and functionality. Elsevier. Current opinion in Biotechnology 18: 176-183.
17. Jing A, Ai-Li L, Ben-Xian S, Xiang-Chen M (2015) Multi-cereal beverage fermented by Lactobacillus Helveticus and Saccharomyces Cerevisie. Journal of food science 80: 1259-1265.

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Fermentation of Rice Flour Supernatant using *Lactobacillus Paracasei CBA L74*

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The concept of functional food has evolved throughout the years. These days we define a food to be functional when it confers a benefit to human health. Lactic acid fermentation is one of the most important and used food processing technologies to produce this type of food. Fermentation of *Lactobacillus Paracasei* CBA L74 - a homofermentative, gram positive probiotic - on rice flour suspension has been proved to be a successful route to produce functional foods. However, the process was characterized by high energy consumption, low air sparging and complex expensive metabolite separation.
A research program has been developed to carry out *L. Paracasei* fermentation using a low viscosity medium based on rice flour suspension. Fractionation of rice flour suspension provided a medium characterized by low amounts of solids and nutrients at concentration high that support the microorganism growth. Fermentations were carried in a 1L batch reactor equipped with an agitation systems designed to provide uniform mixing. The composition of the medium; cells, glucose, protein, pH, metabolite production as well as *L. Paracasei'* growth trend was analysed throughout the process. Fermentation on supernatant obtained from rice flour and water suspension through centrifugation were possible to produce a potential probiotic functional food with a good bacterial charge.

1. Introduction

The concept of functional food has evolved throughout the years. Nowadays consumers do not consume food only to satisfy hunger and provide nutrients but also to prevent nutrition related diseases, improve the overall quality of life, physical health and mental wellbeing (Kotilainen et al., 2006; Robertfroid, 2000a, 2000b).
In Europe food is considered functional if it has beneficial effects on one or more functions of the human organism, together with the basic nutritional impact, thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. (Siro et al., 2008, Diplock et al., 1999)
Probiotics are part of the functional foods widely used to improve health. With the term "probiotics" we usually refer to lactobacilli or bifidobacteria, both dominant endogenous members of the gut microbiota but probiotics are usually defined as live microbial feed supplement which beneficially affects the host by improving and/or modifying its intestinal microbial balance, (Fuller, 1989) without the side effects of traditional antibiotics. The amount of probiotics considered adequate to obtain a temporary colonization of the intestine by lactic acid bacteria is at least 10^7 living cells per day (International dairy federation, 1997). Usually probiotics are given to consumers with fermented products such as yoghurt, fermented vegetables or meats and most products currently available on the market use either Bifidobacteria- dominant inhabitant of the large intestine, or Lactobacillus- dominant inhabitant of the small intestine for their long history of safe use. The ability of the strain to survive during processing, product development, shelf life and the transit through the human gut is essential for its use in commercial products. These characteristics are species and strain specific; for example, Lactobacilli are in general more robust and resistant to low pH respect to Bifidobacteria, therefore lactobacillus species are technological more suitable for applications. (Mättö J. et al., 2006, Ross R.P. et al.,

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2005). These advantages have fuelled the industrial interest in the development of systems for their production and lactic acid fermentation is a bacterial process that takes place during the production of numerous food products, providing characteristic aroma and texture to the final product and conferring beneficial properties for human health. Most studies found in literature are performed on dairy products and cereals and in this paper a promising process on supernatant obtained from an aqueous rice flour suspension is discussed to promote the growing of *Lactobacillus paracasei* CBA L74, a gram positive, homofermentative probiotic with several studies showing various health benefits such as anti- inflammatory effects in coeliac disease interfering with the entrance of gliadin peptide P31-43 (Gallo M., et al, 2019, Sarno et al., 2014), the reduction of common infectious diseases in children attending day-care (Nocerino R. et al., 2017), positive changes in gut microbiota composition (Berni Canani R. et al., 2017) and anti-inflammatory effects against colitis (Zagato et al.,2014). Several fermentation tests were usually performed on synthetic minimal cultures (Van der Osten et al., 1989, Martinez et al 2007, Narendranath et al., 2001) but to the best of our knowledge none were performed on food matrices. The choice to use minimal cultures is usually related to various benefits, reducing feed preparation costs, and in some cases reducing the clogging problems in immobilized cell reactor (Chen et al., 1990).
The aim of the present study was to ferment *L. paracasei* on a matrix that allowed the extraction of the metabolites. The liquid phase separated by centrifugation of rice flour and water suspension was used as fermentation feedstock: the supernatant was a less complex matrix characterized by low content of nutrients. The centrifugation rate was used as tuning variable to modify the fermentation broth.

2. Materials and Methods

2.1 Microorganism

The strain used as starter culture was *Lactobacillus paracasei* CBA L74, (Heinz Italia SpA), International Depository Accession Number LMG P-24778. That strain is a gram positive homofermentative, facultative anaerobic bacteria. Stored at -26 °C in animal free Broth (BAF), revitalized through incubation at 37 °C for 24 hours.

2.2 Apparatus

Fermentation tests were carried out in a batch reactor of 1.5 L. The experimental laboratory system consisted of four components: vessel, mixing system, thermal conditioning system and temperature measurement system. The vessel was cylindrical Pyrex (20 cm high, 10 cm ID) equipped with an external jacket that allowed the circulation of a service fluid necessary to maintain the entire apparatus at a constant temperature (37 °C). The fluid was thermo regulated through a thermo-stated bath. The reactor is shown in Figure 1 (a)
The mixing system was a stainless-steel impeller equipped with two Rushton turbines, as shown in Figure 1 (b): the first turbine with a diameter of 5 cm and a height/ width/ thickness of blades of 1.2/ 1.7/ 0.1 cm respectively; the second turbine had a diameter of 4 cm and height/ width/ thickness of blades of 0.7/1.0/ 0.1 cm respectively That mixing system was used to guarantee homogeneity of the matrix and avoid gradient temperature and microorganism concentration. The impeller was linked to a motor that allowed the adjustment of the stirring speed and had an input connected to a probe, useful for automatic measurement of temperature simultaneously to the process' progress. pH and temperature were continuously measured using InPro 3100i Mettler Toledo probe with a diameter of 12 cm and a length of 15 cm as shown in Figure 1 (c).

(a) (b) (c)

Figure (1): the apparatus used to perform the fermentations; (a) the batch reactor with the external jacket for fluid circulation (b) the impeller with two Rushton turbines, (c) the probe used to measure pH and temperature

2.3 Operating conditions and procedure:

The process was divided in three steps: the first step was the preparation of the initial rice flour and water suspension, the second step was its centrifugation to obtain the supernatant, and the third step was the fermentation of supernatant. All processes were carried out under sterile conditions. The suspension consisting of rice flour (15 %), water (84 %), glucose as an immediate carbon source (1%) was loaded into the reactor (with a working volume of 1 L) and treated with amylase (0.12 %) at pre-set temperature/ time to avoid the gelatinization of the starch and to provide homogeneity of the suspension. The suspension was centrifuged and the supernatant used for successive tests. To assess the best time/centrifugal force ratio to obtain a suspension containing a low concentration of solid particles but sufficient nutrient content to ensure microorganism growth, several combination of centrifugation rate and centrifugation time were investigated. After centrifugation of the suspension we obtained a supernatant of almost 700 mL that was subjected to heat treatment in autoclave (121°C for 20 min) before the start of fermentation to ensure the sterility of the substrate. The fermentation of the supernatant was performed in the system described in §2.2. Tests were carried out at 37 °C for 24 h.

2.4 Analytical Methods

The fermentation process performed was analysed in terms of bacterial growth, glucose concentration, protein content, pH, primary and secondary acids produced. To evaluate *L. paracasei* CBA L74 growth and the presence of contaminants, spread plate method was used. Lactobacillus' growth was evaluated on M.R.S. plates (Sigma Aldrich), whereas MacConkey (Sigma-Aldrich) and Gelisate agars (Biolife), were used to evaluate the presence of contaminants. The plates were incubated then at 37 °C for 2 days before reading. Glucose concentrations during the fermentation process was analysed using a colorimetric method: D-glucose Assay kit (GOPOD format). The protein content in the supernatant initially and throughout the fermentation was analysed using the Bradford assay (Sigma-Aldrich). Lactic acid concentration was measured by using an HPLC (Agilent technologies 1100 with visible UV detector). Agilent Zorbax C18 column (4,6 mm x 150 mm and a pore size of 80 A) was used. The flow rate was set at 0.8 mL/ min; the mobile phase was ammonium phosphate with a pH of 2.7 with detection at 218 nm. Butyric, propionic and acetic acids concentration was carried out by gas chromatography (Agilent Technologies 6890). Capillary Poraplot Q column (25m x 0.32mm) was used. The flow rate was 200 ml\ min. The mobile phase was helium gas. The internal standard is esanoic acid at 5 g/L.

3. Results

The fermentation tests were performed using the previously described system.
Centrifugation carried out under severe centrifugation (high centrifugation rotation rate) deprived the liquid phase of suspended nutrients at such extension that no bacteria growth was recorded on the recovered liquid phase (not reported). According to this observation the maximum centrifugation rate was set at 2000 rpm. Presented results regard two conditions: centrifugation at 1000 rpm for 15 minutes; centrifugation at 2000 rpm for 15 minutes. The two centrifugal speeds gave two matrices different in terms of nutrient content which led to different bacterial growth and production of lactic acid. In Figure 2 is reported the growth obtained during 24 hours of fermentation. As shown, both fermentations started with a bacterial charge of 10^6 CFU/ ml. In the first fermentation condition (1000 rpm) the highest growth was reached at 22 h corresponding to $1.2*10^8 \pm 6.2*10^7$ CFU/ml, while in the second fermenting condition (2000 rpm) the highest charge was reached at 18h corresponding to $2.10^7 \pm 8.5*10^6$ CFU/ ml. pH levels followed the same trend in both conditions, dropping from initial values of 5.2 to 3.47 decreasing as lactic acid production increased. Lactic acid production was also investigated considering it is the primary metabolite of *L. paracasei*. In both conditions its production started after 8 hours of fermentation, and the values reached are reported in figure (6). As shown, the highest amounts were measured at 20 hours at 1000rpm corresponding to 486.225 ± 2.06 ppm, while at 2000 rpm 350 ± 4.13 ppm were registered at 20 hours of fermentation. Secondary acids such as butyric, acetic and propionic acids were not detected throughout the fermentations, further confirming the sterility of the process. The difference on glucose concentration (Figure 4) between the two matrices is not very relevant (2.1mg/mL at 1000 rpm and 2.03 mg/mL at 2000 rpm) and in both cases the quantity was enough to allow the growth. Protein content (Figure 5) on the other hand was much lower in the suspension obtained at the higher centrifugation speed (2000 rpm) that consented a greater separation of nutrients. In fact, such centrifugation speed gave a protein concentration of 0.085 mg/mL as oppose to 0.38 mg/mL in the supernatant obtained at 1000 rpm.

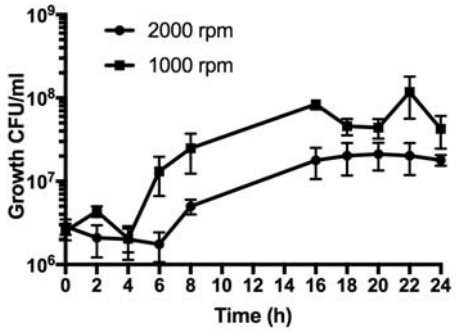

Figure (2): Comparison between the growth of L. paracasei fermented on 1000 rpm supernatant and 2000 rpm supernatant

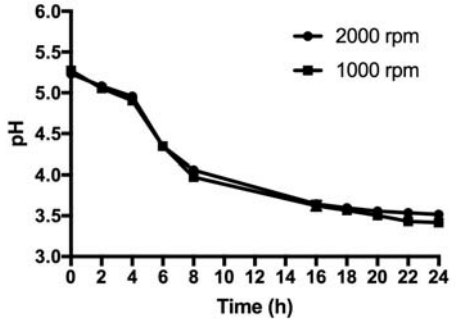

Figure (3): comparison between pH levels during fermentations.

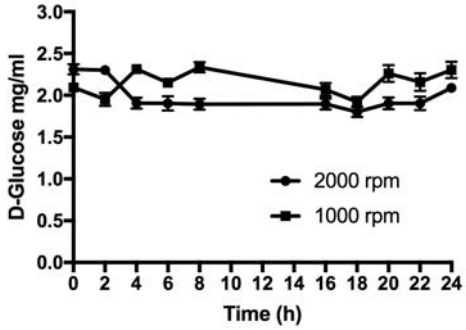

Figure (4): comparison between D-glucose concentrations during fermentations.

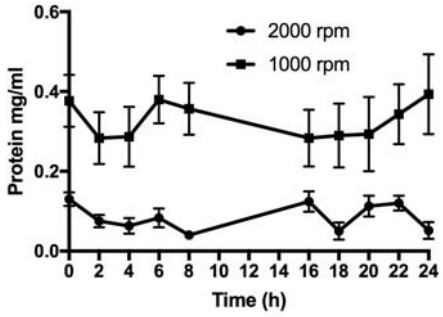

Figure (5): comparison between protein concentration during fermentations.

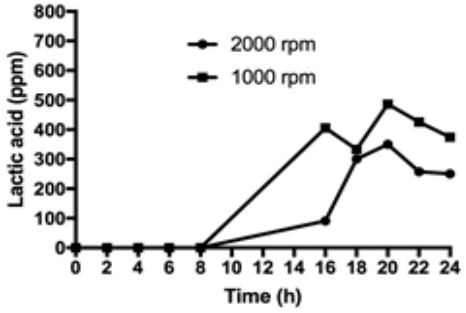

Figure (6): comparison between lactic acids concentration produced during fermentations

4. Conclusions

Both fermentation conditions gave satisfying results in terms of microbial growth and lactic acid production. In the first condition tested (1000 rpm) the lag phase lasted for the first 4 h of fermentation while lasting for 6 hours in the second condition tested (2000 rpm). The exponential phase ended around 16 h for both conditions (1000 rpm and 2000 rpm) with a higher bacterial charge for 1000 rpm (respectively 8.39 $*10^7$ CFU/ ml and $5*10^6$ CFU/ ml). Death phase wasn't detected in both cases considering the tests only lasted for 24 hours and no sampling was done afterwards indicating its start after 24 h. pH levels dropped constantly in both cases from a value of 5.2 to a more acidic level around 3.6 which was consistent with the maximum lactic acid production of about 350 ppm at 2000 rpm (20 h) and about 480 ppm at 1000 rpm (20 h). Regarding glucose and protein concentrations, in both conditions they remained constant during the entire fermentation process even if in the supernatant obtained at 2000 rpm the mean value of proteins was lower than that at 1000 rpm probably due to the stronger centrifugal force. In particular, regarding glucose concentration an explanation could be the fact that the microorganism can use other carbon sources for the growth, while for proteins the constant concentration during the whole process could be explained by a simultaneous consumption and synthesis of proteins, which is not discriminated by the method of analysis.

The aim of this study was testing the possibility of a potential fermentation process on supernatant obtained from a rice flour and water suspension containing minimal amounts of nutrients. The choice of supernatant was made to allow energy savings as the viscosity is similar to water, as well as an easier pre-treatment of the sample for analysis. The lactic acid bacteria chosen was *L. paracasei* CBA L 74 (depositary accession number LMG P-24778) provided by the Kraft-Heinz company. Two fermentation conditions were studied; supernatant obtained after centrifugation of suspension at 1000rpm and 2000 for 15 minutes in both cases. Fermentation tests done confirmed overall better conditions in the case of supernatant 1000 rpm, providing a higher bacterial charge and a maximum lactic acid concentration at earlier times. Future prospects include understanding the interaction of such obtained probiotics with the digestive system and possible methods of transit / release through the digestive system.

References

Berni Canani R., De Filippis F., Nocerino R., Laiola M., Paparo L., Calignano A., De Caro C., Coretti L., Chiariotti L., Gilbert J. A., Chiariotti L., Ercolini D., 2017, Specific signatures of the gut microbiota and increased levels of butyrate in children treated with fermented cow's milk containing heat-killed Lactobacillus paracasei CBA L74. Applied and environmental microbiology, American society of microbiology, vol 83, issue 19, e01206-17.

Chen C., Dale M.C. and Okos M.R., 1990, Minimal nutritional requirements for immobilized yeast. Biological industries. Vol 36 P:993-1001

Diplock A. T., Aggett P. J., Ashwell M., Bornet F., Fern E. B., & Roberfroid M. B., 1999.,Scientific concepts of functional foods in Europe: Consensus document, British journal of nutrition, 81(Suppl. 1), S1–S27.

Fao/Who Food and agriculture organization of the United Nations, World Health Organization. 2001, Evaluation of health and nutritional properties of probiotics in food Including powder milk with live lactic acid bacteria. C´Ordoba. 34 P.

Fuller, 1989, Probiotics in man and animal, Journal of applied Bacteriology. 66, 365-378

Gallo M., Nigro F., Passannanti F., Nanayakkara M., Lania G., Parisi F.,Salameh D., Budelli A., Barone M.V., Nigro R., 2019, Effect of pH control during rice fermentation in preventing gliadin P31-43 in epithelial cells,International journal of food sciences and nutrition, Doi 10.1080/09637486.2019.1599827,1465-3478

Koletzko B, Aggett Pj, Bindels Jg. 1998. Growth, development and differentiation: A functional food science approach. Brazilian journal of Nutrition, 80:35–45

Kotilainen L, Rajalahti R, Ragasa C, Pehu E., 2006, Health enhancing foods: Opportunities for strengthening the sector in developing countries. Agriculture and rural development discussion paper 30.

Martinez A., Grabar T.B., Shanmugam K.T., Yomano L.P., York, S.W., Ingram, L.O.,2007, Low salt medium for lactate and ethanol production by recombinant Escherichia Coli B, Biotechnological Letters 29:397-404. Doi10.1007/S10529-006-9252-Y.

Mättö J., Alakomi H., Vaari A., Virkajärvi and Saarela M., 2006, Influence of processing conditions on Bifidobacterium animalis subsp. Lactis functionality with a special focus on acid tolerance and factors affecting it, International dairy journal 16,1029-1037.

Narendranath N.V., Thomas K.C. and Ingledew W.M., 2001, Effects of acetic acid on the growth of Saccharomyces Cerevisiae in a minimal medium. Journal of industrial microbiology and biotechnology, Vol 26, P:171-177

Nocerino R., Paparo L., Terrin G., Pezzella V., Amoroso A., Cosenza L., Cecere G., De Marco G., Micillo M., Albano F., Nugnes R., Ferri P., Ciccarelli G., Giaccio G., Spadaro R., Maddalena Y., Berni Canani F., Berni Canani R., 2017, Cow's milk and rice fermented with Lactobacillus paracasei CBA L74 prevent infectious diseases in children: A randomized controlled trial, Clincal Nutrition.;36(1):118-125.

Robertfroid, M. B., 2000a, Concepts and strategy of functional science: the European perspective. American journal of clinical nutrition, 71, 1660e1664.

Robertfroid, M. B., 2000b. A European consensus of scientific concepts of functional foods. Nutrition, 16, 689e691

Ross R.P., Desmonf C., Fitzgerald G.F. and Staton C., 2005, Overcoming the technological hurdles in the development of probiotic foods, Journal of applied microbiology, 98, 1410-1417.

Sarno M., Lania G., Cuomo M., Nigro F., Passannanti F., Budelli A., Fasano F., Troncone R., Auricchio S., Barone M. V., Nigro R., Nanayakkara M., 2014, Lactobacillus paracasei CBA L74 interferes with gliadin peptides entrance in Caco-2 cells, International Journal of food sciences and nutrition, 65 (8),pp.953-959. https://doi.org/10.3109/09637486.2014.940283

Sanders Me., 2003, Probiotics: Considerations for human health, Nutrition reviews 61:91–9.

Siro, Istvan, Et Al.,2008 Functional food. Product development, marketing and consumer acceptance—A review. Appetite 51.3, 456-467.

Van Der Osten C.H., Gioannetti C., Sinskey J. 1989, Design of a defined medium for growth of Corynebacteriium Glutamicum in which citrate facilitates iron uptake, Biotechnology Letters Vol 11, No1, P 11-16.

Zagato E, Mileti E, Massimiliano L, Fasano F, Budelli A, Penna G, et al.,2014, Lactobacillus paracasei CBA L74 metabolic products and fermented milk for infant formula have anti- inflammatory activity on dendritic cells in vitro and protective effects against colitis and an enteric pathogen in vivo. PLoS One; 9:1e14

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Effect of pH control during rice fermentation in preventing a gliadin P31-43 entrance in epithelial cells

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ABSTRACT

Coeliac disease is an increasingly recognised pathology, induced by the ingestion of gluten in genetically predisposed patients. Undigested gliadin peptide can induce adaptive and innate immune response that unleash the typical intestinal mucosal alterations. A growing attention is paid to alternative therapeutic approaches to the gluten-free diet: one of these approaches is the use of probiotics and/or postbiotics. We performed lactic fermentation of rice flour with and without pH control, using *Lactobacillus paracasei CBA L74* as fermenting strain. We evaluated bacterial growth, lactic acid production during fermentation and gliadin peptide P31-43 entrance in CaCo-2 cells with and without pH control. When pH control was applied no differences were observed in terms of bacterial growth; on the contrary, lactic acid production was greater, as expected. Both samples could inhibit the P31-43 entrance in CaCo-2 cells but the effect was significantly greater for samples obtained when the pH control was applied.

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Introduction

Food is no longer regarded only as a source of energy and vehicle of essential nutrients for the body, but also as an instrument able to beneficially influence some biological functions and/or the risk of disease development (Menrad 2003). "Functional food" is a vast and heterogeneous group of food products that have the capacity to bring benefits to the body (Roberfroid 2002) (Diplock et al. 1999) (Huggett and Verschuren 2009). It can be food as it is (such as soy, whole grains, fruits, vegetables) or food modified by technologies such as fermentation (Hasler 2002). Fermented food could be of great help to people with a food-related disease (Rubio-Tapia et al. 2013). Coeliac disease, a food induced disease, is an increasingly widespread intestinal autoimmune disease (Grace-Farfaglia 2015), caused by an innate and adaptive immune response in genetically predisposed individuals. This response is induced by specific peptides of gliadin, a food protein present in wheat and other cereals (barley and rye). To understand the complexity of coeliac disease, we must remember that there are numerous gliadin peptides, classifiable in toxic and immunogenic peptides (Ciccocioppo et al. 2005), which are responsible for an immune response in genetically predisposed individuals. Furthermore, that peptide can enter the intestinal mucosa through two different ways, paracellular (Fasano 2012) and transcellular (Barone et al. 2010). In particular, the entry of the gliadin P31-43 and P57-68 peptides within the enterocytes is responsible for a powerful immune response. Those peptides can enter CaCo-2 cells and intestinal enterocytes *via* endocytosis, both receptor and raft mediated, and can increase intracellular Ca++ (Barone et al. 2011; Caputo et al. 2010, 2013). Recent data have shown that gliadin peptides, in particular peptide P31-43, but not P57-68, act as growth factors for cell lines such as CaCo-2 cells, are able to induce proliferation of coeliac crypt enterocytes (Barone et al. 2007; Barone et al. 2011) and have other biological effects, such as increase of innate immunity and inflammation markers and alteration of intracellular trafficking (Zimmer et al. 2010; Barone et al. 2014; Barone and Zimmer 2016). Several *in vivo* and *in vitro* studies have demonstrated the beneficial effect that some functional foods, such as probiotics and their supernatant or fermented food, presumably containing postbiotics, have on coeliac disease (Sarno

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et al. 2014; Klemenak et al. 2015; Quagliariello et al. 2016; Francavilla et al. 2018). Sarno et al. (2014) highlighted a possible way to use supernatants obtained by cereal fermentations to inhibit the entrance of gliadin peptides in enterocytes. It seems that in the presence of products coming from the fermentation of cereal substrates with *Lactobacillus paracasei* (LP), it is possible to slow down the entry of the P31-43 peptide within the enterocytes. Furthermore, from the literature, we knew that the pH value during the lactic fermentation could influence the biomass concentration, the yield and fermentation time (Beal and Corrieu 1991; Amrane and Prigent 1999; Adamberg et al. 2003). This study has the aim to verify if pH control during rice fermentation (Gallo et al. 2018) could improve not only the bacterial growth or the lactic acid production, but could also prevent the P31-43 entrance in intestinal epithelial cell line. In this article, we show that pH control during fermentation of *LP CBA L74* could improve the effect on P31-43 entrance, respect to the not controlled. Moreover, the effect on P31-43 entrance was retained in a < 10 KDa fraction of the supernatant.

Material and methods

Strain

The strain used as starter culture was *LP CBA L74* (Heinz Italia SpA), International Depository Accession Number LMG P-24778.

That strain is a Gram-positive, facultative heterofermentative, oxygen-tolerant anaerobic bacteria. It was stored in a freeze-dried form, at −20 °C and revitalised in an animal-free broth (20 g/l Bacto Yeast Extract, BD Biosciences, Milan, Italy; 0.5 g/l $MgSO_4$, Sigma-Aldrich, Milan, Italy; 50 g/l Glucose, Sigma-Aldrich; 0.5 g/l citric acid, Sigma-Aldrich). The revitalisation lasted 24 h at 37 °C.

Rice fermentation

Rice flour was provided by HJ Heinz (Heinz Italia SpA, Latina, Italy). A suspension of 15% of rice flour (w/v) in water, with 2% w/v of added glucose was fermented by *LP CBA L74* (Heinz Italia SpA), International Depository Accession Number LMG P-24778. The inoculum was composed of 9 mL animal free broth with a mean bacterial charge of 10^8 CFU/ml. The fermentation was performed without and with pH control (using NaOH (0.2 M), fed by a peristaltic pump, the pH value was maintained at 5.8) and lasted 24 h at 37 °C. Aseptic sampling was performed after the inoculum (t_0) and after 2 (t_2), 4 (t_4), 6 (t_6), 8 (t_8), 14 (t_{14}), 16 (t_{16}), 18 (t_{18}), 20 (t_{20}), 22 (t_{22}) and 24 (t_{24}) h of fermentation. Each sample was used for pH measurements and for microbiological and chemical analysis. In particular, after serial dilutions, samples were sowed on petri plate, prepared with MRS agar (Oxoid, Basingstoke, UK); to assess the microbiological growth the lactobacilli were incubated for 48 h at 37 °C, in anaerobic conditions, guaranteed by the use of special anaerobic kits (Anaerogen Compact, Oxoid). The in line pH analysis was possible through the use of the transmitter M300 (Mettler Toledo, Columbus, OH), connected to the In Pro 3100 probe (Mettler Toledo), that was inserted into the reactor through an input present on the impeller. pH control was performed through the use of the same device, connected *via* a peristaltic pump to the NaOH tank. The amount of lactic acid produced during rice fermentation with LB was determined by High Performance Liquid Cromatography (HPLC) (Agilent Technologies 1100, Santa Clara, CA), equipped with a column C18 (Agilent Zorbax C18), with UV detector. The eluent was 0.1 M $NH_4H_2PO_4$, at a flow rate of 0.8 ml/min. Lactic acid and bacterial growth were analysed as markers of the fermentation trend.

Culture substrates

For the treatment of the cells, the fermented samples have to undergo to a freeze-drying procedure. For this reason, the fermented samples, collected at the end of the process (24 h), were heated at 85 °C for 20 s, to inactivate the live bacteria, and then were freeze-dried, to obtain powder containing bacterial bodies and fermentation products. The control powders consisted of rice flour processed with a similar procedure but without the inoculation step. The freeze drying step was necessary because was not possible to put directly the fermented samples in contact with cells.

The freeze-dried samples (fermented and control) were diluted in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, San Giuliano Milanese, Italy) and stirred at 37 °C for about 15 min. The solution obtained was centrifuged at 3000 rpm for 10 min at room temperature to recover the supernatant necessary for cell contact.

Cell cultures

CaCo-2 cells were grown in DMEM (GIBCO, San Giuliano Milanese, Italy), 10% foetal bovine serum (FBS) (GIBCO) and 1 mM glutamine in an incubator

at a temperature of 37 °C and 5% CO_2 for 2/4 d after towing.

Fluorescence assay

Cells, grown on sterile glass coverslips, were transferred into a 24-well plate and treated with the supernatant for 30 min. After this time, two different trials were performed. One consisting in the treatment of CaCo-2 cells with lissaminated P31-43 in 5% CO_2 atmosphere at 37 °C, for 30 min. The second trial was identical to the first one, but performed after a robust washing by PBS, in order to verify a possible memory effect on the cells due to the supernatant treatment.

Peptides were used in the following concentrations: P31-43-lissamine at 20 µg/ml; unlabelled P31-43 peptides were used at 80 µg/ml. After washing in PBS, the coverslips were briefly fixed (5 min) with paraformaldehyde 3% (Sigma-Aldrich, Milan, Italy) at room temperature. The coverslips, after mounting on glass slides, were observed by confocal microscope (LSM 510 Zeiss, Milan, Italy), and images were analysed with AIS Zeiss software to evaluate the intensity of fluorescence (FI) of the microscopic field under consideration (Barone et al. 2001; Monaco et al. 2001; Gargiulo et al. 2007; Lombardi et al. 2008). Magnification of the micrographs was the same for all the figures shown (63 × objective).

Both gliadin peptide P31-43 and P31-43 linked to fluorochrome Lissamine (wavelength 575/595) were sinthetyzed by InBios, Napoli, Italy. The peptides, obtained by Ultrasart-D20 (Sartorius AG, Goettingen, Germany) filtration, were 95% pure, as analysed by MALDI-tof. P31-43 sequence was LGQQQPFPPQQPY.

Supernatant fractions

LP CBA L74 supernatants were fractionated using Amicon® Ultra-15 10 K Centrifugal Filter Devices (Millipore, Milano, Italy), according to the manufacture instructions. Briefly, 15 ml of supernatant were added to the Amicon® Ultra filter device and centrifugation (4000 rpm for 40 min) was performed to separate the fractions. Both <10 and >10 KDa were used as before.

Statistical analysis

Statistical analysis and graphics were obtained from GraphPad Prism (San Diego, CA). Mean and standard deviation of the experiment was calculated; their significance was evaluated by Student's *t*-test accepting as significant only results that showed values of $p < 0.05$.

Results

Rice fermentation: with and without pH control

pH values, LP growth curve and lactic acid production for the two conditions tested (with and without pH control) are shown in Figure 1. In the first case, where pH was not controlled (Figure 1(A)) its value dropped from 5.8 ± 0.035 to a final value of 3.5 ± 0.057. In the second case, the pH value was maintained around 5.8 until the end of the fermentation. Lactobacillus' growth for the two conditions tested is shown in Figure 1(B). For both conditions, the fermentation started with an initial charge of about $2*10^6$ ($2.647*10^6 \pm 7.9*10^5$ CFU/ml for fermentations performed with no pH control and $2.57*10^6 \pm 2.88*10^6$ for fermentations performed without pH control). A first difference after 4 h can be observed: when no pH control was adopted, no substantial differences were present respect to the initial charge (Lactobacillus' charge at t_0 was $2.647*10^6$ CFU/ml, while after 4 h was $2.08*10^6 \pm 4.2*10^4$ CFU/ml); with pH control, after 4 h, a lower charge than that initial was found (Lactobacillus' charge at t_0 was $2.57*10^6$ CFU/ml, while after 4 h it was $4.33*10^5 \pm 9.2*10^4$ CFU/ml)) and this difference was statistically significant. However, no significant difference between the two fermentation runs was notable from 16 h until the end of the process at t_{24} (t_{24} charge of fermentations without pH control: $5.41*10^8 \pm 3.8*10^8$ CFU/ml; t_{24} charge of fermentations with pH control: $3.16*10^8 \pm 1.7*10^8$ CFU/ml). The different phases of the microbial growth were identified for both conditions (with and without pH control): the lag, exponential and stationary phases lasted, respectively, from 0 to 4, from 4 to 14 and from 14 to 24 h when no pH control was applied; while the lag, exponential and stationary phases in case of pH control, lasted respectively, from 0 to 6, from 6 to 18 and from 18 to 24 h. To monitor the fermentation process we evaluated the lactic acid production (Figure 1(C)). As expected lactic acid was produced during fermentation in both conditions tested (with and without pH control). From 14 h until the end of the fermentation significant differences were observed between the two types of fermentations (at t_{14} the concentration of lactic acid produced without pH control was 625 mg/l; while that produced at t_{14} with pH control was 1283 mg/l; t_{20} concentration of lactic acid produced during fermentation without pH control was 1300 mg/l; whereas that for the fermentation with

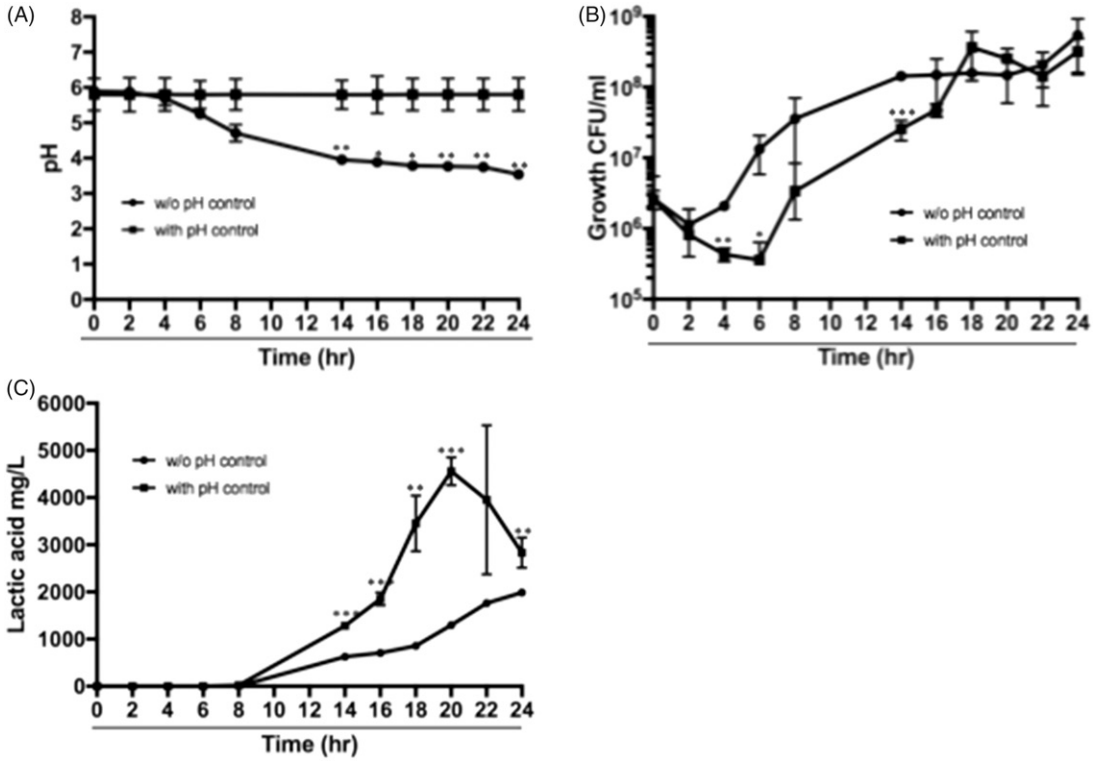

Figure 1. Fermentation results at different sampling times (t0, t2, t4, t6, t8, t14, t16, t18, t20, t22, t24). (A) pH values. (B) Microbial growth value (CFU/ml). (C) Lactic acid concentrations (mg/L). Circles represent rice samples obtained when no pH control was applied during fermentation; squares represent rice samples obtained when pH control was applied during fermentation. Each result was the mean value of a triplicate analysis. Student t-test. $*p < .05$; $**p < .01$; $***p < .001$.

pH control the concentration was 4500 mg/l). When the pH was controlled, at t_{24}, the lactic acid concentration was reduced up to a value of 2835 mg/l. This concentration was in any case higher than that observed when no pH control was applied (1987 mg/l), and the difference was statistically significant.

P31-43 entrance after supernatant treatment

We treated Caco-2 cells with gliadin peptide, P31-43, conjugated with the fluorochrome Lissamine (P31-43-liss), to follow its entrance into the cells (Barone et al. 2010; Zimmer et al. 2010; Barone et al. 2011; Reinke et al. 2011). Initially, we treated CaCo-2 cells with the supernatant obtained by the fermented rice flour (with and without pH control), and after 30 min of soaking, we added the peptide P31-43-liss, keeping it in contact with cells for 30 min, as schematised in Figure 2(A). The peptide entrance in CaCo-2 cells was fully visible in large and numerous vesicles with a cytosolic distribution, as shown in Figure 2(B), CTR panel. Treatment with the two fermented rice flours induced a significant reduction of the P31-43-liss entrance, as is also shown in Figure 2(B). Interestingly, the fermented rice obtained with pH control was more efficient (−49%) than the fermented rice obtained without pH control (−26%), and the difference was statistically significant (Figure 2(C)).

The ability of the supernatant obtained by the fermented rice flour (with and without pH control) to prevent P31-43-liss entrance after PBS wash of the cells was also investigated, as schematised in Figure 3(A). As shown in Figure 3(B,C), both treatments, with and without pH control, were able to prevent P31-43-liss entrance after washing (−40 and −28%, respectively). Again, also in this case, the fermented supernatant obtained with pH control was more efficient in reducing P31-43-liss entrance, and the difference was statistically significant. Interestingly, fractionations of the *LP CBA L74* supernatants (<10 and >10 KDa) showed that the activity on P31-43 entrance was retained by the <10 KDa, as shown in Figure 4.

Discussion

Considering the increase in the incidence of diseases such as coeliac disease (Fasano et al. 2003), there is a growing interest in alternative therapies to the gluten-free diet. The oral enzyme administration (Mitea et al. 2007), the tissue transglutaminase inhibition (Wolf

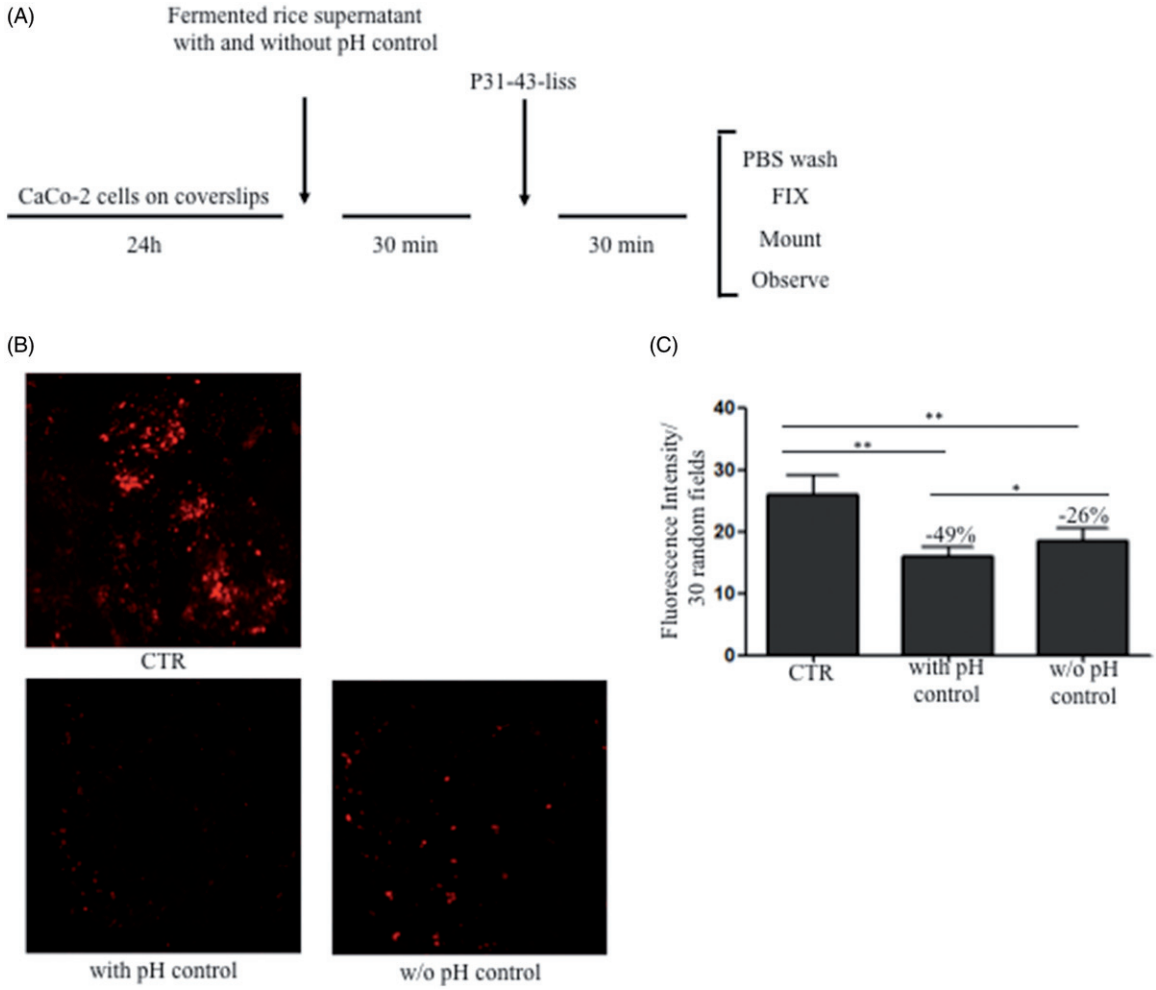

Figure 2. Rice fermented with and without pH control by LP CBA L74, reduced P31-43-liss entrance in CaCo-2 cells. (A) Schematic representation of treatments with P31-43-liss and fermented rice (obtained with or without pH control) added at the same time to CaCo-2 cells. (B) Immunofluorescence images of CaCo-2 cells treated for 30 min with P31-43-liss after treatment with fermented rice for 30 min. Representative images. (C) Statistical analysis of FI in CaCo-2 cells treated with P31-43 and after treatment with fermented rice. Fluorescence intensity was valuated in 30 random fields for each sample. Columns indicate mean and bars standard deviation of five independent experiments done in triplicate. Student t-test. $^{*}p < .05$; $^{**}p < .01$.

et al. 2011), the inhibition of HLA-DQ2 mediated antigen presentation (Xia et al. 2006) are all examples of experimental alternative ways of coeliac disease treatment. In other cases, the attention was directed to the product: Greco et al. (2011), for example, demonstrated how a hydrolysed wheat flour was not toxic to patients with CD. In a prior study, Sarno et al. (2014) showed how *LP CBA L74*, its supernatant and fermented cereals could interfere with the gliadin peptide entrance in CaCo-2 cells. Based on this, the aim of this work was to investigate if pH control during fermentation process of rice with probiotic *LP CBA L74*, could improve not only the bacterial growth and the lactic acid production, but also the ability to interfere P31-43 entrance in the CaCo-2 cells, and also if these effects persisted after washing away the supernatant. PH control during the rice fermentation process greatly improved the lactic acid production, as expected (Amrane and Prigent 1999). Afterwards, we compared the effect of the two types of processes on P31-43-liss entrance in CaCo-2 cells. In the previous study of Sarno et al. (2014) was evident a difference between different fermented cereals in inhibiting P31-43 entrance in CaCo-2 cells, showing how the rice, fermented with *LP CBA L74*, was the more efficient in preventing the gliadin peptide entrance. In this article, we used the same assay to analyse the effect of pH control on *LP CBA L74* growth respect to no control of pH, using rice as substrate. We confirmed that fermented rice supernatant was able to prevent P31-43 entrance in CaCo-2 cells and showed that control of the pH during fermentation can improve this activity. Moreover, we tested whether these effects persisted after washing away the supernatants. Both rice

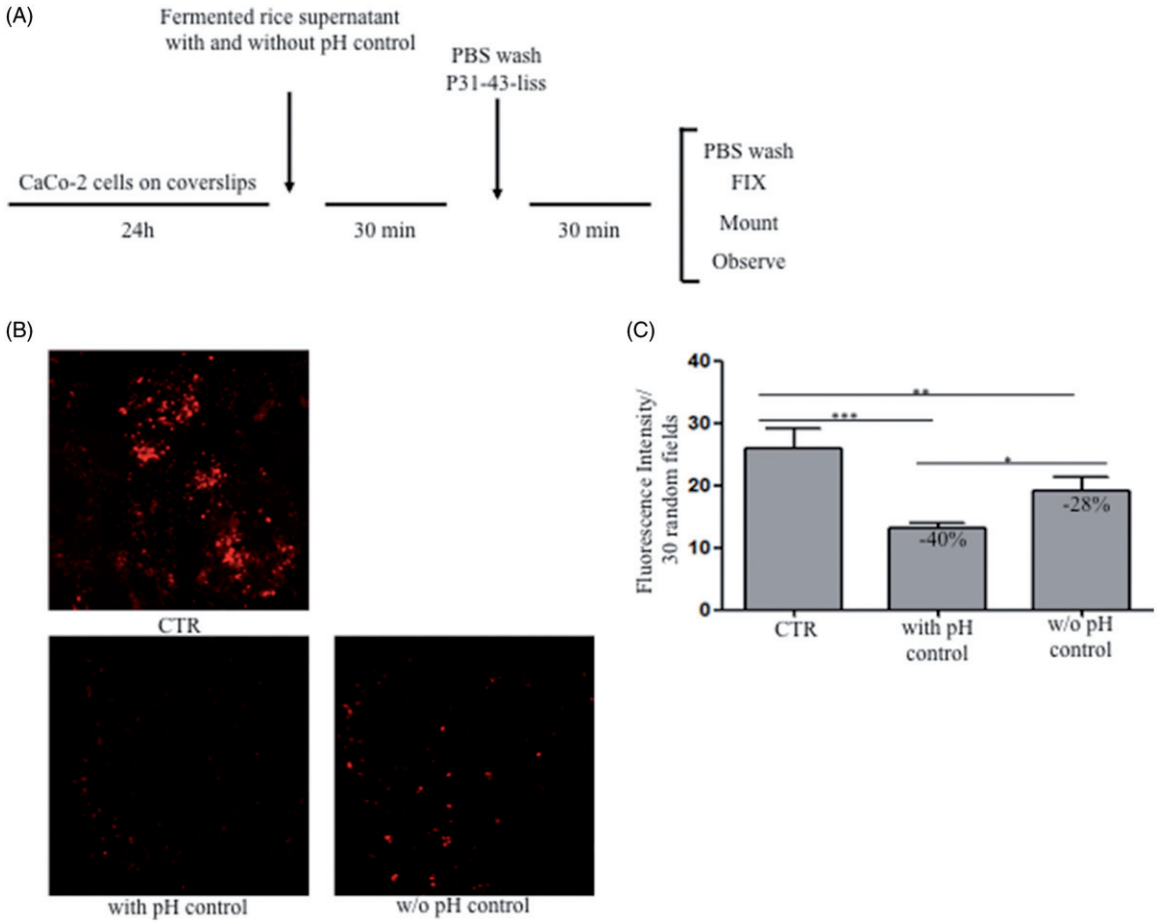

Figure 3. After PBS washing, rice fermented with and without pH control by LP CBA L74, was still able to reduce P31-43-liss entrance in CaCo-2 cells. (A) Schematic representation of treatments with P31-43-iss and fermented rice (obtained with or without pH control) added sequentially to CaCo-2 cells. (B) Immunofluorescence images of CaCo-2 cells treated for 30 min with fermented rice, PBS washed and then treated with P31-43-liss for 30 min. Representative images. (C) Statistical analysis of FI in CaCo-2 cells treated with P31-43 and after treatment with fermented rice. Fluorescence intensity was valuated in 30 random fields for each sample. Columns indicate mean and bars standard deviation of five independent experiments done in triplicate. Student t-test. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

fermented with and without pH control, were able to prevent P31-43-liss entrance even after they were washed away from the cells, triggering into the cells a sort of memory that could prevent the entrance of P31-43-liss. Also, in this case, the product obtained with pH control was more efficient than that obtained without pH control, in preventing P31-43-liss entrance. This information could be useful in the field of nutrition; in fact, it suggests that the effect of certain probiotics/postbiotics could last also after they have left the intestine. The compounds/metabolites contained in the supernatant responsible for this effect have not been isolated yet. In a preliminary experiment, we tested <10 and >10 KDa supernatant fractions ability to prevent P31-43-liss entrance in the CaCo-2 cells and found that the effect was present only in the lower fraction (<10 KDa) indicating that the effect could be due to a small peptide. Small peptides produced by fermentation with a functional value have been described also in other contests: Gonzalez et al. (2013) for example described the production of bioactive peptides during milk fermentation by a *Bifidobacterium bifidum*; Takano (1998) showed how peptides with hypertension reduction effect could be produced during milk fermentation. In addition to this, several studies demonstrated the importance of pH on the bacterial metabolic pathways (Zhu and Yang 2004), in particular on protein synthesis (Lee et al. 2008; Wu et al. 2011; Zhai et al. 2014). So it is possible to postulate that the improved ability of the rice obtained with controlled pH fermentation to prevent P31-43 entrance could depend on a more efficient protein/peptide synthesis. On the other hand, an altered protein metabolism, in the uncontrolled pH fermentation, could be the cause of the lower ability to interfere with the peptide entrance. Furthermore,

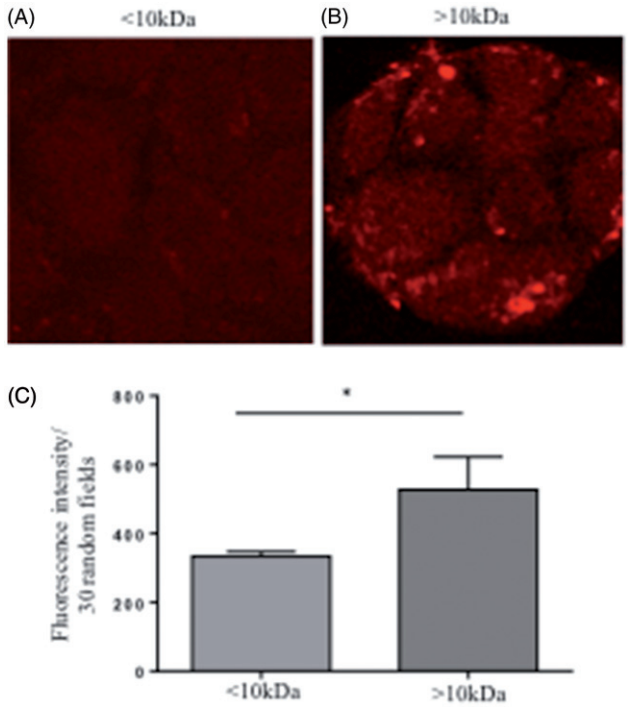

Figure 4. <10 and > 10 KDa fractionament of LP CBA L74 supernatant on P31-43-liss entrance in CaCo-2 cells. Only <10 KDa fractions of LP CBA L74 supernatant reduced P31-43-liss entrance in CaCo-2 cells. (A) Immunofluorescence images of Caco-2 cells treated for 30 min with P31-43-liss after treatment with < 10 (A) and > 10 KDa (B) fractions of LP CBA L74 supernatants for 30 min. Representative images. (C) Statistical analysis of FI in Caco-2 cells treated with P31-43liss after treatment with LP CBA L74. Fluorescence intensity was valuated in 30 random fields for each sample. Columns indicate mean and bars standard deviation of five independent experiments done in triplicate. Student t-test. $^{*}p < .05$.

the fermentation pH seems one of the most important factors affecting the peptide content in the fermented substrate; in fact, the peptide content increases significantly if the pH values are controlled (Aasen et al. 2000; Bautista-Expósito et al. 2018). On the same way, Herranz et al. (2001), studying several pH values to maintain constant during fermentation, found the best one to obtain the highest bacteriocin production. Considering the reduced biological effect – pH related – found in this study for *LP CBA L74* rice fermentations, future perspective could be the study of the *LP* viability through a simulated digestive process and changes of its activity on CaCo-2 cells; in fact, in the stomach the probiotic is subjected to a very low pH, which could reduce viability and activity, including protein synthesis. In an attempt to improve and preserve *LP CBA L74* activity during rice fermentations, an interesting approach could be the encapsulation of probiotic cells to protect them from low pH value and digestive enzymes.

Conclusion

The ability of *LP CBA L74* in producing some functional compounds, already investigated by Sarno, was confirmed and the evaluated biological effect appeared to be affected also by some parameters, useful in the control of the fermentation process, as the pH control is. Novelty of our work was not in the fermentation method in itself (pH control is in fact widely applied, also in industrial processes) rather in understanding that by controlling the pH during fermentation we had an impact on the results already obtained by Sarno, on the entry of the P31-43 peptide into CaCo-2 cells. We know in fact that the effect is due to some metabolite/s produced during fermentation; therefore, our goal was to verify if the pH control could influence the production of this metabolite/s. Alternative therapies to gluten-free diet in CD have been proposed. Either focussed on the destruction of gliadin peptides present in food, or by blocking the entry of peptides in the intestinal epithelium, preventing the activation of the immune response. Probiotics and/or its supernatant and/or fermented foods have characteristics that could be useful in both these areas.

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Roberto Nigro, Maria Vittoria Barone and Marianna Gallo designed the research; Giuliana Lania, Merlin Nanayakkara, Federica Nigro, Francesca Parisi, Francesca Passannanti, and Dana Salameh, performed the experiments and collected and analysed the data; Marianna Gallo, Federica Nigro and Francesca Passannanti contributed to the interpretation of the results; Andrea Budelli provided the raw materials and *Lactobacillus paracasei CBA L74*; Marianna Gallo, Federica Nigro and Francesca Passannanti wrote the article and have the primary responsibility for the final content. All authors read and approved the final manuscript.

Ethical review

This study does not involve any human or animal testing.

Informed consent

Written informed consent was obtained from all study participants.

Disclosure statement

Dr Andrea Budelli is currently employed by Heinz BV, Netherlands. He provided the raw materials (rice flour) and *Lactobacillus Paracasei CBA L74* and participated in the design of the study. He did not have any additional role in the data collection and analysis, decision to publish, or preparation of the manuscript.

Heinz BV did not provide any financial support to the authors for the experimental activity, did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Aasen IM, Møretrø T, Katla T, Axelsson L, Storrø I. 2000. Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. Appl Microbiol Biotechnol. 53(2):159–166.

Adamberg K, Kask S, Laht TM, Paalme T. 2003. The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. Int J Food Microbiol. 85(1–2): 171–183.

Amrane A, Prigent Y. 1999. Differentiation of pH and free lactic acid effects on the various growth and production phases of *Lactobacillus helveticus*. J Chem Technol Biotechnol. 74(1):33–40.

Barone MV, Gimigliano A, Castoria G, Paolella G, Maurano F, Paparo F, Maglio M, Mineo A, Miele E, Nanayakkara M, et al. 2007. Growth factor-like activity of gliadin, an alimentary protein: implications for coeliac disease. Gut. 56(4):480–488.

Barone MV, Nanayakkara M, Paolella G, Maglio M, Vitale V, Troiano R, Ribecco MTS, Lania G, Zanzi D, Santagata S. 2010. Gliadin peptide P31-43 localises to endocytic vesicles and interferes with their maturation. PLoS One. 5(8):e12246.

Barone MV, Sepe L, Melillo RM, Mineo A, Santelli G, Monaco C, Castellone MD, Tramontano D, Fusco A, Santoro M. 2001. RET/PTC1 oncogene signaling in PC Cl 3 thyroid cells requires the small GTP-binding protein Rho. Oncogene. 20(48):6973–6982.

Barone MV, Troncone R, Auricchio S. 2014. Gliadin peptides as triggers of the proliferative and stress/innate immune response of the celiac small intestinal mucosa. Int J Mol Sci. 15(11):20518–20537.

Barone MV, Zanzi D, Maglio M, Nanayakkara M, Santagata S, Lania G, Miele E. 2011. Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking. PLoS One. 46:e17039.

Barone MV, Zimmer KP. 2016. Endocytosis and transcytosis of gliadin peptides. Mol Cell Pediatr. 3(1):8.

Bautista-Expósito S, Peñas E, Silván JM, Frias J, Martínez-Villaluenga C. 2018. PH-controlled fermentation in mild alkaline conditions enhances bioactive compounds and functional features of lentil to ameliorate metabolic disturbances. Food Chem. 248:262–271.

Beal C, Corrieu G. 1991. Influence of pH, temperature, and inoculum composition on mixed cultures of Streptococcus thermophilus 404 and *Lactobacillus bulgaricus* 398. Biotechnol Bioeng. 38(1):90–98.

Caputo I, Barone MV, Lepretti M, Martucciello S, Nista I, Troncone R, Auricchio S, Sblattero D, Esposito C. 2010. Celiac anti-tissue transglutaminase antibodies interfere with the uptake of alpha gliadin peptide 31-43 but not of peptide 57-68 by epithelial cells. Biochim Biophys Acta. 1802(9):717–727.

Caputo I, Lepretti M, Secondo A, Martucciello S, Paolella G, Sblattero D, Barone MV, Esposito C. 2013. Anti-tissue transglutaminase antibodies activate intracellular tissue transglutaminase by modulating cytosolic Ca2+ homeostasis. Amino Acids. 44(1):251–260.

Ciccocioppo R, Di Sabatino A, Corazza GR. 2005. The immune recognition of gluten in coeliac disease. Clin Exp Immunol. 140(3):408–416.

Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB. 1999. Scientific concepts of functional foods in Europe: consensus document. Br J Nutr. 81:S1–S27.

Fasano A, Berti I, Gerarduzzi T, Not T, Colletti RB, Drago S, Elitsur Y, Green PHR, Guandalini S, Hill ID, et al. 2003. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. Arch Intern Med. 163(3):286–292.

Fasano A. 2012. Zonulin, regulation of tight junctions, and autoimmune diseases. Ann N Y Acad Sci. 1258(1):25–33.

Francavilla R, Piccolo M, Francavilla A, Polimeno L, Semeraro F, Cristofori F, Castellaneta S, Barone M, Indrio F, Gobbetti M, et al. 2018. Clinical and microbiological effect of a multispecies probiotic supplementation in celiac patients with persistent IBS-type symptoms: a randomized, double-blind, placebo-controlled, multicenter trial. J Clin Gastroenterol. 53:e117–e125.

Gallo M, Nigro F, Passannanti F, Salameh D, Budelli A, Marzocchella A, Nigro R. 2018. Rice fermentation by *Lactobacillus paracasei* CBAL74. Int J Rice Res. DOI: 10.29011/ IJRR-103. 000003

Gargiulo A, Auricchio R, Barone MV, Cotugno G, Reardon W, Milla PJ, Ballabio A, Ciccodicola A, Auricchio A. 2007. Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement. Am J Hum Genet. 80(4):751–758.

Gonzalez CG, Gibson T, Jauregi P. 2013. Novel probiotic-fermented milk with angiotensin I-converting enzyme inhibitory peptides produced by Bifidobacterium bifidum MF 20/5. Int J Food Microbiol. 167(2):131–137.

Grace-Farfaglia P. 2015. Bones of contention: bone mineral density recovery in celiac disease-a systematic review. Nutrients. 7(5):3347–3369.

Greco L, Gobbetti M, Auricchio R, Di Mase R, Landolfo F, Paparo F, Di Cagno R, De Angelis M, Rizzello CG, Cassone A, et al. 2011. Safety for patients with celiac disease of baked goods made of wheat flour hydrolyzed during food processing. Clin Gastroenterol Hepatol. 9(1):24–29.

Hasler CM. 2002. Functional foods: benefits, concerns and challenges-a position paper from the american council on science and health. J Nutr. 132(12):3772–3781.

Herranz C, Martínez J, Rodríguez J, Hernández P, Cintas L. 2001. Optimization of enterocin P production by batch fermentation of Enterococcus faecium P13 at constant pH. Appl Microbiol Biotechnol. 56(3–4):378–383.

Huggett AC, Verschuren PM. 2009. Research needs for establishing the safety of functional foods. Nutr Rev. 54(11):S132–S140.

Klemenak M, Dolinšek J, Langerholc T, Di Gioia D, Mičetić-Turk D. 2015. Administration of Bifidobacterium breve decreases the production of TNF-α in children with Celiac Disease. Dig Dis Sci. 60(11):3386–3392.

Lee KB, Lee HG, Pi KB, Choi YJ. 2008. The effect of low pH on protein expression by the probiotic bacterium *Lactobacillus reuteri*. Proteomics. 8(8):1624–1630.

Lombardi M, Castoria G, Migliaccio A, Barone MV, Di Stasio R, Ciociola A, Bottero D, Yamaguchi H, Appella E, Auricchio F. 2008. Hormone-dependent nuclear export of estradiol receptor and DNA synthesis in brest cancer cells. J Cell Biol. 182(2):327–340.

Menrad K. 2003. Market and marketing of functional food in Europe. J Food Eng. 56(2–3):181–188.

Mitea C, Havenaar R, Drijfhout JW, Edens L, Dekking L, Koning F. 2007. Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. Gut. 57(1):25–32.

Monaco C, Visconti R, Barone MV, Pierantoni GM, Berlingieri MT, De Lorenzo C, Mineo A, Vecchio G, Fusco A, Santoro M. 2001. The RFG oligomerization domain mediates kinase activation and re-localization of the RET/PTC3 oncoprotein to the plasma membrane. Oncogene. 20(5):599–608.

Quagliariello A, Aloisio I, Bozzi Cionci N, Luiselli D, D'Auria G, Martinez-Priego L, Pérez-Villarroya D. 2016. Effect of Bifidobacterium breve on the intestinal microbiota of coeliac children on a gluten free diet: a pilot study. Nutrients. 8(10):pii: E660.

Reinke Y, Behrendt M, Schmidt S, Zimmer KP, Naim HY. 2011. Impairment of protein trafficking by direct interaction of gliadin peptides with actin. Exp Cell Res. 317(15):2124–2135.

Roberfroid MB. 2002. Global view on functional foods: European perspectives. Br J Nutr. 88(S2):S133–S138.

Rubio-Tapia A, Hill ID, Kelly CP, Calderwood AH, Murray JA. 2013. ACG clinical guidelines: diagnosis and management of celiac disease. Am J Gastroenterol. 108(5):656–676.

Sarno M, Lania G, Cuomo M, Nigro F, Passannanti F, Budelli A, Fasano F. 2014. *Lactobacillus paracasei* CBA L74 interferes with gliadin peptides entrance in Caco-2 cells. Int J Food Sci Nutr. 65:953–959.

Takano T. 1998. Milk derived peptides and hypertension reduction. Int Dairy J. 8(5–6):375–381.

Wolf J, Lachmann I, Wagner U, Osman AA, Mothes T. 2011. Quantification of human tissue transglutaminase by a luminescence sandwich enzyme-linked immunosorbent assay. Anal Biochem. 419(2):153–160.

Wu R, Zhang W, Sun T, Wu J, Yue X, Meng H, Zhang H. 2011. Proteomic analysis of responses of a new probiotic bacterium *Lactobacillus casei* Zhang to low acid stress. Int J Food Microbiol. 147(3):181–187.

Xia J, Siegel M, Bergseng E, Sollid LM, Khosla C. 2006. Inhibition of HLA-DQ2-mediated antigen presentation by analogues of a high affinity 33-residue peptide from alpha2-gliadin. J Am Chem Soc. 128(6):1859–1867.

Zhai Z, Douillard FP, An H, Wang G, Guo X, Luo Y, Hao Y. 2014. Proteomic characterization of the acid tolerance response in *Lactobacillus delbrueckii subsp. bulgaricus* CAUH1 and functional identification of a novel acid stress-related transcriptional regulator Ldb0677. Environ Microbiol. 16(6):1524–1537.

Zhu Y, Yang ST. 2004. Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. J Biotechnol. 110(2):143–157.

Zimmer KP, Fischer I, Mothes T, Weissen-Plenz G, Schmitz M, Wieser H, Büning J, Lerch MM, Ciclitira PC, Weber P, Naim HY. 2010. Endocytotic segregation of gliadin peptide 31–49 in enterocytes. Gut. 59(3):300–310.

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Lactic Fermentation of Cereal Flour: Feasibility Tests on Rice, Oat and Wheat

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Abstract

Background and objective: Consumers show increasing interests in probiotic foods and lactic acid fermentations. Cereal flour, can be a good fermentable substrate due to its prebiotic nature; from which, synbiotic products can be prepared. The aim of the current study was to investigate if three various cereal flours rice, oat and wheat would be good potentially functional foods.

Material and methods: Fermentation tests were carried out on rice, oat and wheat flours, using *Lactobacillus paracasei* CBA L74 and 1.5-L fermenter with 1-L working volume. After 24 h, microbial growth, pH value, lactic acid production and starch consumption were assessed.

Results and conclusion: In all three flours, pH reduction was seen; particularly in rice flour. The highest Lactobacillus growth and lactic acid production were achieved at the end of rice fermentation. The greatest starch consumption was reported at the end of rice fermentation. In conclusion, lactic fermentation of cereals as potentially functional foods was possible for the three flours. However, the best result belonged to rice flour.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

In industrial countries, roles of foods have progressively been changed. In the past, the term of nutrition referred to disciplines that simply aimed at ensuring adequate calorie intakes. Nowadays, nutrition can be considered as a tool to prevent various diseases. Further attentions have been paid to foods and food ingredients, resulting in a significant development of the so-called functional foods. A functional food can be described as a food with additional functions, often linked to health promotion or disease prevention. Particular attentions are now paid to probiotics, the live microorganisms with health benefit on hosts when supplied in adequate quantities [1]. The most studied and used bacteria within the probiotics include lactic acid bacteria (LAB) and bifidobacteria [2]. Based on the literatures, the minimum quantity to achieve a temporary colonization of the intestine by a lactic fermenting strain includes at least 10^7 CFU g^{-1} [3]. Currently, the most well-known and marketed functional foods are dairy products [4]. However, non-dairy products, especially cereals, are becoming preferred vehicles for probiotics [5-7]. Cereal-based functional foods represent a new frontier due to their vast resources and ease of storage, compared to other products from various sources [8]. Experimental evidence prove good growth of LAB in cereal matrices, suggesting that the incorporation of probiotic strains into cereal substrates results in production of fermented foods with characteristics that can contribute to the development of consumers' well-being [9-12]. Cereal flour is a good

example of fermentable substrates because of its composition. Indeed, cereal flour is a rich source of nutrients such as resistant starches, water soluble and insoluble β-glucans and arabinoxylans as well as indigestible oligosaccharides that make cereal flours good prebiotics. A prebiotic is described as an indigestible food ingredient that beneficially affects the host by selectively stimulating growth and/or activity of one or more resident bacterial species in colon and thus improves host health [13]. Fermented cereal flour can be considered as a symbiotic; in which, probiotic and the prebiotic effects are present. Therefore, lab-scale tests were carried out on submerged fermentations of cereal flours using *Lactobacillus* (*L.*) *paracasei* CBA L74. The aim of the current study was to investigate feasibility of the fermentation, especially on cereal flours and direct use of these food products as functional foods. Another aim was to prepare a semi-finished product to introduce in production process of the functional foods. Various cereal flours of rice, oat and wheat were used to investigate effects of cereal characteristics such as starch chemical-physical properties, protein and mineral concentration and nutritional values on fermentation. The differences are verified for the Lactobacillus growth, pH values and lactic acid production (the main fermentation product) as the strain is homo-fermentative.

2. Materials and methods

2.1 Strain

The strain used in starter culture included *L. paracasei* CBA L74, (Heinz, Italia SpA) with the International Depository Accession Number of LMG P-24778. That strain was a Gram-positive homo-fermentative, facultative anaerobic bacteria. The bacterial strain was stored in freeze-dried form at -20°C and recovered at 37°C in 0.9% sodium chloride solution, 10 min before fermentation tests. The inoculum bacterial density included 10^8 CFU ml^{-1}.

2.2 Rice, oat and wheat flours

Rice, oat and wheat flours were provided by Heinz, Italia SpA. The starch content of the flours included 67.0, 60.0 and 46.7% for rice, wheat and oat, respectively. Before each fermentation process, flour was subjected to heat treatment (120°C for 90 min) to decrease possible microbial loads.

2.3 Lab-scale fermentation bioreactors

The experimental lab-scale bioreactors for fermentations included four components of a vessel and system of mixing, thermal conditioning and pH and temperature measure-ments. The vessel, with a maximum capacity of 1.5 l, was a cylindrical Pyrex (20 cm high, 10 cm ID) equipped with an external jacket that allowed circulation of a service fluid necessary to preserve a constant temperature in the entire apparatus. The fluid was thermos-regulated through a thermo-controlled water bath (Haake, USA) set at 37°C. The mixing system consisted of a stainless steel impeller, having an inclined blade and a Rushton impellers connected to a motor (a three-phase asynchronous electric motor of 0.25 Hp; 0.18 kW; 1310 rpm with a speed reducer of 170-880 rpm) that allowed adjustment of the stirring speed and hence axial and radial mixings. Therefore, efficient mixing and good homogeneity of the substrates were achieved, using mixing speed of 180 rpm and food dye; as demonstrated by preliminary mixing tests (not shown). Briefly, the best mixing speed (180 rpm) was considered as a speed that allowed achieving a perfect distribution of the food dye in the suspension; mostly within 10 min. The vessel, the mixing system and the entire mechanism used for the lab-scale experiments are shown in Figs. 1A, B and C, respectively. A Mettler Toledo device equipped with an autoclavable In Pro 3100 Probe (Mettler Toledo, USA) was used for the continuous measurement of pH and temperature.

Figure 1. The vessel, mixing system and bioreactor. A) The vessel was built using Pyrex and equipped with an external jacket that allowed the circulation of service fluid necessary to maintain the entire apparatus at a constant temperature. B) The stainless steel impeller with two various types of turbines: a blade turbine to reach an axial flow and a Rushton turbine to reach a radial flow. C) The complete equipment was used for the lab-scale experiments.

2.4 Lab-scale fermentation protocols

Submerged fermentation was carried out using 1.5-L fermenter with 1-L working volume. The first step included sterilization of the fermenter and mixing system

at 121°C for 20 min, using autoclave. Then, 150 g of each flour (rice, oat and wheat flours), previously sanitized by dry heat, were mixed in 0.850 l of sterilized distilled water under laminar flow hood to avoid contamination. The mixture was tyndallized using two consecutive cycles of heating (90°C) and cooling (37°C) to decrease the microbial load. The fermentation temperature was controlled at 37°C under aerobic conditions and the fermentation run was stopped after 24 h. This protocol was used for the three flours.

2.5 Analytical methods

To characterize the fermentation process, a small quantity of the substrate was aseptically collected from the bioreactor at specific sampling times (t_0, t_4, t_{14}, t_{20}, t_{24}; intended as hours from the inoculum phase). These samples were used for microbiological and chemical analyses. After serial diluting, substrate was spread on Petri dishes of MRS agar (Oxoid, UK) and incubated at 37°C for 48 h under anaerobiosis, using Anaerogen Compact anaerobic kits (Oxoid, UK). To investigate the presence of contaminants, samples were spread on Petri dishes prepared with PCA bacteriological agar, yeast extract agar, peptose peptone, glucose, McConkey agar and gelatine peptone agar (Oxoid, UK).

The quantity of lactic acid produced during fermentation was assessed using a high performance liquid chromatography (Agilent Technologies 1100, USA), equipped with a C18 column (Agilent Zorbax, 4.6 × 150 mm, pore size of 80 A°) and a UV detector. The eluent included 0.1 M of $NH_4H_2PO_4$ with a flow rate of 0.8 ml min^{-1}. The mobile phase included ammonium phosphate with a pH of 2.7 and detection at 218 nm. The temperature of analysis included 30°C. Secondary acid products such as butyric and acetic acids were assessed using gas chromatography (Agilent Technologies 6890, USA) equipped with a Capillary Poraplot Q column (25 m × 0.32 mm). The flow rate included 200 ml min^{-1} and the mobile phase included helium gas. The starch content in raw materials and fermented samples was assessed using Total Starch Assay Kit (AA/AMG) (Megazyme, Ireland) through spectrophotometric analysis at 510 nm.

2.6 Statistical analysis

Each test was carried out in triplicate. Statistical analysis was carried out using GraphPad Prism 7.0a (San Diego, CA, USA). Mean and standard deviation of the experiments were calculated and their significance was evaluated using student's t-test. The significant was reported when values $P \leq 0.05$.

3. Results and discussion

Although dairy foods are the most common substrates used for the probiotic production, cereal based foods offer a valid alternative. In this study, three flours of rice, oat and wheat were fermented. The *L. paracasei* CBA L74 was used as fermenting culture. This was a homo-fermentative bacterial strain with lactic acid final product. To test the flour fermentation, the lactic acid concentration, pH values (for increased production of the lactic acid, lower pH levels were expected), *L. paracasei* growth and starch consumption were assessed. The aim of the present work was to investigate feasibility of lactic acid fermentation of cereal flours as potentially functional foods. Based on the literatures, a minimum level of 10^6 viable probiotic bacteria per milliliter or gram of the food product was considered sufficient [14]. The pH results from the fermentation of the three flours (rice, oat and wheat) are shown in Fig. 2.

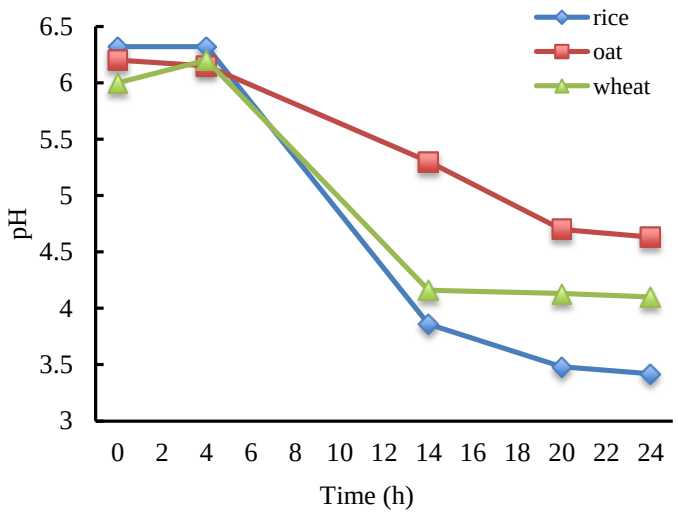

Figure 2. The pH values for the three flours tested at various sampling times (t_0, t_4, t_{14}, t_{20}, t_{24}). Blue, red and green lines represented results for rice, oat and wheat, respectively. Each result was the mean value of a triplicate analysis using student t-test ($P<0.001$).

The t_0 value of pH was near 6 in the three flours. After a lag time of nearly 4 h; in which, the pH value remained almost constant for the three flours, pH progressively decreased during the fermentation time reaching at t_{24}, values of 3.42 ±0.055, 4.63 ±0.050 and 4.1 ±0.060 for rice, oat and wheat, respectively. Each result was the mean value of triplicate fermentations. The lactobacilli growth on the three flours is shown in Fig. 3. For all the flours, a lag phase of about 4 h could be assessed with a limited growth of the Lactobacillus.

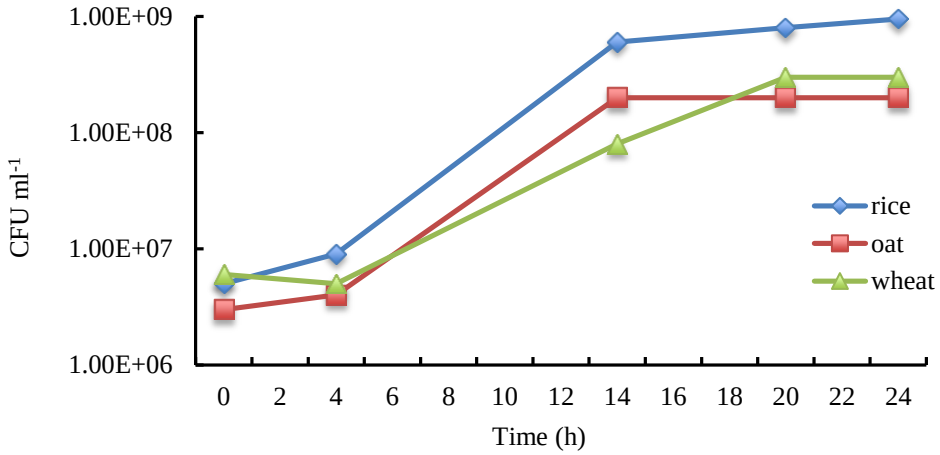

Figure 3. Growth rates (CFU ml^{-1}) for the three flours. Blue, red and green lines represented results for rice, oat and wheat, respectively. Each result was the mean value of a triplicate analysis using student t-test (P<0.001).

Various growth rates could be calculated for these cereals. The exponential phase for rice shifted from t_4 to t_{14} with a viable LAB concentration of $6\times10^8 \pm 1.5\times10^8$ CFU ml^{-1}; after which, a stationary phase could be observed up to the end of fermentation process (t_{24}) with $9.5\times10^8 \pm 1.3\times10^8$ CFU ml^{-1}. The exponential phase for oat shifted from t_4 to t_{14} with $2\times10^8 \pm 7\times10^7$ CFU ml^{-1}. This remained almost constant until the end of fermentation. The period of lag phase for wheat was similar other cereals, while the exponential phase was longer (nearly 6 h). This was continued to t_{20} with a LAB concentration of $3\times10^8 \pm 1.7\times10^8$. Concentration of viable Lactobacillus remained constant until t_{24}. The lactic acid quantity is shown in Fig. 4. No lactic acid was detected at the beginning of fermentation. An almost linear production rate of lactic acid was detected after lag phase until the end of fermentation. Values of 3100 ± 380, 2000 ± 130 and 2300 ± 143 mg l^{-1} were measured for rice, oat and wheat, respectively. No secondary metabolites (butyric and acetic acids) were detected using gas chromatography analysis, proving absence of the contaminants.

Starch content of the substrates during the fermentations is shown in Fig. 5. Starting with an initial starch concentration of 100.5 ± 2.5, 69 ± 4 and 90 ± 4 g l^{-1} for rice, oat and wheat flours respectively, a progressive consumption was found over the time. For oat and wheat flours, decreases in starch content were almost linear. For rice flour, a significant decrease was seen until t_{20}; after which, the concentration was almost constant. The final concentrations of starch included 30 ± 4, 50 ± 4.3 and 57 ± 5 g l^{-1} for rice, oat and wheat flours respectively, corresponding to consumptions of 70, 28 and 37% of the initial starch contents. Results for the rice, oat and wheat fermentations are summarized in Table 1.

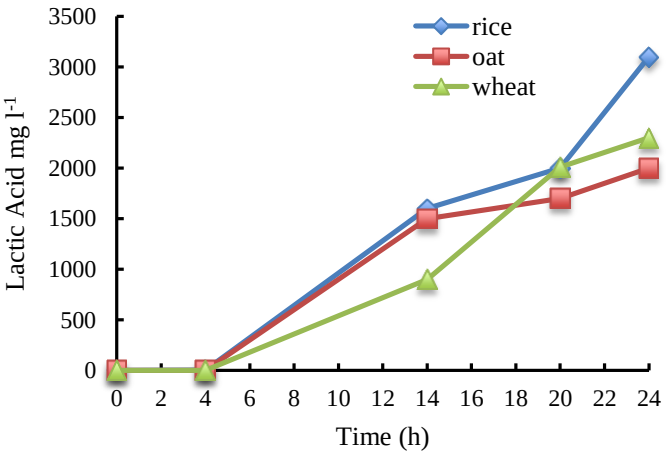

Figure 4. Lactic acid production (mg l^{-1}) during the fermentation of the three flours. Samples included no lactic acids at t0 min. Concentrations after 24 h included 3100 mg l^{-1} for rice, 2000 mg l^{-1} for oat and 2300 mg l^{-1} for wheat. Blue, red and green lines represented results for rice, oat and wheat, respectively. Each result was the mean value of a triplicate analysis using student t-test (P<0.001).

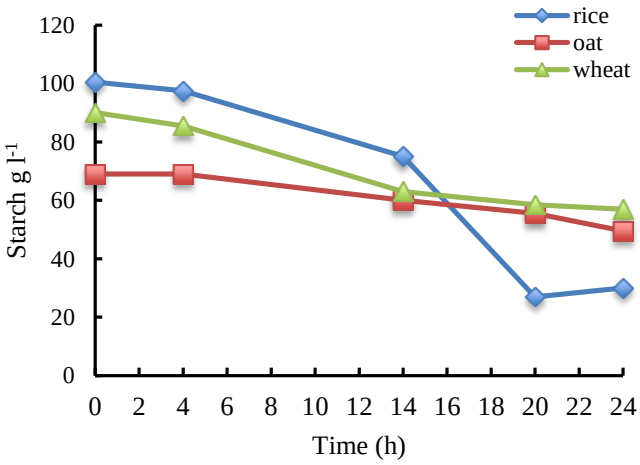

Figure 5. Starch concentrations (g l^{-1}) for the three flours. Blue, red and green lines represented results for rice, oat and wheat, respectively. Each result was the mean value of a triplicate analysis using student t-test (P<0.001).

Table 1. Summary of the results for rice, oat and wheat at t_0 and t_{24} fermentations

	Time [h]	Rice	Oat	Wheat
pH	0	6.32 ± 0.13	6.24 ± 0.04	6.00 ± 0.25
	24	3.42 ± 0.05	4.63 ± 0.05	4.10 ± 0.06
Bacterial density [CFU ml^{-1}]	0	5.00E+06 ± 5.29E+05	3.00E06 ± 8.19E05	6.00E+06 ± 8.19+05
	24	9.50E+08 ± 1.30E+08	2.07E+08 ± 6.81E+07	3.00E+08 ± 6.08E+07
Lactic acid [mg l^{-1}]	0	0	0	0
	24	3100 ± 380	2000 ± 130	2300 ± 143
Starch [g l^{-1}]	0	100.50 ± 2.50	69 ± 4	90 ± 4
	24	30 ± 4	50 ± 4.30	57 ± 5

Concentrations of the starch following fermentation could be still high in a food product for human consumption. Since the major aim of the current study was to test the feasibility of cereal fermentation to obtain a semi-finished product for using in other foods, the highlighted concentrations of starch might not be considered high. The greatest *L. paracasei* growth and starch consumption were seen at the end of rice fermentation. This could be due to several factors. First, rice starch granules included the smallest size [15] and small granules were hydrolyzed more rapidly than larger ones using α-amylase; as suggested by Vasanthan and Bhatty [16]. This could make rice starch more accessible to hydrolysis than oat and wheat starches. Second, high amylose starches were particularly resistant to hydrolysis [17]. It seemed that rice, containing amylose of 21-25% w w^{-1} on total starch dry basis [18], included smaller amylose contents than oat (amylose content of 27.5-29.8% w w^{-1} on total starch dry basis) [19] and wheat (amylose content of 25.6% w w^{-1} on total starch dry basis) [18]. These data of granule size and amylose content suggested an easier hydrolysis of the rice starch, compared to that of oat and wheat starches. Regarding lactic acid, the highest concentration was found for the rice flour (3100 mg l^{-1}). Significantly lower levels were reported for the oat (2000 mg l^{-1}) and wheat (2300 mg l^{-1}) flours. This could be linked to the growth of Lactobacillus. Several studies were carried out on cereal fermentation. In most of these studies, a spontaneous fermentation was studied under uncontrolled conditions [20-22]. In other studies, simultaneous saccharification and fermentation were carried out using hydrolytic enzymes [23,24]. Marko et al. [25] studied static fermentations of various cereal suspensions such as wheat flour in water using *L. plantarum* as the fermenting strain. A lag phase longer than that of the present study (8 instead of 4 h) was seen. Starch concentration was lower and starch consumption and lactic acid production were smaller than those found in the current study. In contrast, a similar Δ growth of nearly three logs was reported by Marko et al. [25] and Charalampopoulos et al. [8]. Rice and wheat flours were used as substrates in another study [26]. Fermentation was carried out under static condition, anaerobiosys and pH control. The substrate for fermentation was composed of a simple broth added with various concentrations of pure starch or cereal flour. Results showed greater starch consumption and lactic acid production for rice and wheat flours, compared to that results of the present study did in significantly longer time (6 days instead of 24 h).

4. Conclusion

Based on the current findings, rice seemed the best cereal for the fermentation because of a greater growth, lactic acid production and starch consumption by lactobacilli; possibly due to the granule characteristics of the rice starch. Although oat and wheat seemed to be good substrates for the fermentation, their fermentation process needed optimization. For example, this optimization could be carried out by adding a further available source of carbon such as glucose. In all the fermented substrates, a sufficient bacterial density to consider the products as potential functional foods was achieved. However, In vitro and In vivo experiments are necessary to demonstrate functionality. Fermented flours can be used as semi-finished products in various foods (e.g. baby foods) to further develop their properties such as immunomodulatory activity or digestibility. A future perspective could be process optimization of oat and wheat fermentations using glucose. Furthermore, the process could be improved by controlling pH value during the fermentation and/or by modifying the atmosphere to decrease the O_2 concentration. Study on various flour mixes could be scheduled to verify if each flour alone could affect other flours in terms of microbial growth and lactic acid production.

Contributions

Roberto Nigro and Marianna Gallo designed the research; Federica Nigro, Francesca Passannanti, Dana Salameh and Paola Schiattarella carried out the research; Andrea Budelli provided the raw materials and *L. paracasei* CBA L74; and Marianna Gallo and Francesca Passannanti drafted the manuscript. Marianna Gallo has the primary responsibility for the final content. All authors read and approved the final manuscript.

5. Acknowledgements

Dr Andrea Budelli is currently employed by Heinz BV, Netherlands. He provided the raw materials (rice, oat and wheat flours) and *L. paracasei* CBA L74 and participated in design of the study. He did not have any additional roles in collection and analysis of data, decision of publishing or preparation of the manuscript.

Heinz BV did not provide any financial support to the authors for the experimental activity and did not have any additional roles in the study design, data collection and analysis, publishing decision or manuscript preparation.

6. Conflict of interest

The authors declare no conflict of interest.

References

1. Probiotics in food. Health and nutritional properties and guidelines for evaluation. FAO food and nutrition paper 85. Rome, 2006.
2. Kociubinski G, Salminen S. Probiotics: Basis, State of the Art and Future Perspectives. Functional Food network general meeting. Turku, Finland, March, 8-10, 2006.
3. Khedkar DC, Khedkar GD, Chavan NN, Kalyankar SD, Shrikant. Fermented Milks : Nutritional, Therapeutic Properties, In: Encyclopedia of Food Sciences and Nutrition, Edition: Second. Publisher: Elsevier and Academic Press, London .2003: pp.6230-6235
4. Leatherhead food international. The international market for functional foods, 3rd ed. Functional food market report, 2006.
5. Lamsal BP , Faubion JM. The beneficial use of cereal and cereal components in probiotic foods. Food Rev Int. 2009; 25: 103-114.
doi: 10.1080/87559120802682573
6. Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JAF. Functional foods and nondairy probiotic food development: Trends, concepts and products. Compr Rev Food Sci Food Saf. 2010; 9: 292-302.
doi: 10.1111/j.1541-4337.2010.00110
7. Prado FC, Parada JL, Pandey A , Soccol CR. Trends in non dairy probiotic beverages. Food Res Int. 2008; 41: 111-123.
doi: 10.1007/s13197-015-1795-2
8. Charalampopoulos D, Wang R, Pandiella SS, Webb C. Application of cereals and cereal components in functional foods: A review. Int J Food Microbiol. 2002; 79: 131-141.
doi: 10.1016/S0168-1605(02)00187-3
9. Gallo M, Nigro F, Passannanti F, Salameh D, Budelli A, Marzocchella A, Nigro R. Rice fermentation by *Lactobacillus paracasei* CBA L74. Int J Rice Res. 2018; 2018: 1-5 .
doi: 10.29011/IJRR-103. 000003
10. Salovaara H. Lactic Acid Bacteria: Microbiology and Functional Aspects. Marcel Dekker. New York, 1998.
11. Sarno M, Lania G, Cuomo M, Nigro F, Passannanti F, Budelli A, Fasano F, Troncone R, Auricchio S, Barone MV, Nigro R, Nanayakkara M. *Lactobacillus paracasei* CBA L74 interferes with gliadin peptides entrance in Caco-2 cells. Int J Food Sci Nutr. 2014; 65(8): 953-959
doi: 10.3109/09637486.2014.940283
12. Gallo M, Nigro F, Passannanti F, Nanayakkara M, Lania L, Parisi F, Salameh D, Budelli A, Barone MV, Nigro R. Effect of pH control during rice fermentation in preventing a gliadin P31-43 entrance in epithelial cells. Int J Food Sci Nutr. 2019; 10: 1-9
doi: 10.1080/09637486.2019.1599827
13. Gibson GR , Roberfroid MB. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J Nutr. 1995; 125(6): 1401-1412.
doi: 10.1093/jn/125.6.1401
14. Lacroix C, ildrim S. Fermentation technologies for the production of probiotics with high viability and functionality. Curr Opin Biotechnol. 2007; 18(2): 176-183.
doi: 10.1016/j.copbio.2007.02.002
15. Tester RF, Qi X, Karkalas J. Hydrolysis of native starches with amylases. Anim Feed Sci Technol. 2006: 130: 39-54.
doi: 10.1016/j.anifeedsci.2006.01.016
16. Bhatty T, Vasanthan RS. Physicochemical properties of small and large-granule starches of waxy, regular and high-amylose barleys. Cereal Chem. 1996; 73: 199-207.
doi: 10.1016/S0008-6215(00)00292-5
17. Gallant D, Bouchet B, Buleon A, Perez S. Physical characteristics of starch granules and susceptibility to enzymatic degradation. Eur J Clin Nutr. 1992; 46: S3-S16.
18. Alcazar Alay SC, Almeida Meireles MA. Physicochemical properties, modifications and applications of starches from different botanical sources. Food Sci Technol Campinas. 2015; 35(2): 215-236.
doi: 10.1590/1678-457X.6749
19. Tester RF, Karkalas J. Swelling and gelatinization of oat starches. Cereal Chem. 1996; 73(2): 271-277.
20. Oyewole OB. Lactic fermented foods in Africa and their benefits. Food Control. 1997; 8: 289-297
doi: 10.1016/S0956-7135(97)00075-3
21. Jorgensen H, Sholly D, Oyan Pedersen A, Canibe N, Bach Knudsen KE. Fermentation of cereals-Influence on digestibility of nutrients in growing pigs. Livest Sci. 2010; 134(13): 56-58
22. Baye K, Mouquet Rivier C, Icard Verniere C, Rochette I, Guyot JP. Influence of flour blend composition on fermentation kinetics and phytate hydrolysis of sourdough used to make injera. Food Chem. 2013; 138: 430-436
doi: 10.1016/j.foodchem.2012.10.075
23. Tanaka T, Hoshina M, Tanabe S, Sakai K, Ohtsubo S, Taniguchi M. Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation. Bioresour Technol. 2006; 97: 211-217
doi: 10.1016/j.biortech.2005.02.025

24. Watanabe M, Makino M, Kaku N, Koyama M, Nakamura K, Sasano K. Fermentative L-(+)-lactic acid production from non- sterilized rice washing drainage containing rice bran by a newly isolated lactic acid bacteria without any additions of nutrients. J Biosci Bioeng. 2013; 115: 449-452
doi: 0.1016/j.jbiosc.2012.11.001

25. Marko A, Rakicka M, Mikusova L, Valik L, Sturdik E. Lactic acid fermentation of cereal substrates in nutritional perspective. Int J Res Chem Environ. 2014; 4: 80-92

26. Vishnu C, Seenayya G ,Reddy G. Direct fermentation of various pure and crude starchy substrates to L(+) lactic acid using *Lactobacillus amylophilus* GV6. World J Microbiol Biotechnol. 2002; 18: 429-433
doi: 10.1023/A:1015526221744

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تخمیر لاکتیکی آرد غلات: آزمون‌های امکان‌سنجی بر روی برنج، جو دوسر و گندم

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چکیده

سابقه و هدف: مصرف کنندگان تمایل رو به‌افزایشی را برای غذاهای زیست‌یار[۱] و تخمیر لاکتیکی نشان می‌دهند. آرد غلات، به‌دلیل ماهیت کمک زیست‌یاری[۲] می‌تواند ماده اولیه مناسبی برای تخمیر باشد که از آن می‌توان فرآورده‌های فرزیست‌یار[۳] را تولید کرد. هدف مطالعه حاضر این است که آیا سه نوع آرد غلات شامل برنج، جو دوسر و گندم می‌توانند مواد غذایی فراسودمند بالقوه‌ای باشند یا خیر.

مواد و روش ها: آزمون‌های تخمیر آرد برنج، جو دوسر و گندم با استفاده از *لاکتوباسیلوس پاراکازئی* CBA L74 و فرمانتور ۱/۵ لیتری با حجم کاری یک لیتر انجام شد. پس از ۲۴ ساعت، رشد میکروبی، میزان pH، تولید اسید لاکتیک و مصرف نشاسته تعیین شد.

یافته‌ها و نتیجه‌گیری: در هر سه نوع آرد به‌ویژه آرد برنج کاهش pH مشاهده شد. بالاترین رشد لاکتوباسیلوس و تولید اسید لاکتیک در پایان تخمیر برنج به‌دست آمد. بیشترین میزان مصرف نشاسته در پایان تخمیر برنج گزارش شد. در نتیجه، تخمیر لاکتیکی غلات، به‌عنوان مواد غذایی بالقوه فراسودمند برای این سه نوع آرد امکانپذیر است. اگرچه، بهترین نتیجه به آرد برنج تعلق داشت.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- آرد غلات
- تخمیر لاکتیکی
- *لاکتوباسیلوس پاراکازئی* CBA L7
- زیست‌یار

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- Probiotic [۱]غذاها یا مکمل‌هایی که به دلیل داشتن ریزاندامگان‌ها قادر به بازسازی یا تغییر گیاگان میکربی روده هستند و در حفظ سلامت انسان مؤثرند.
- Prebiotic [۲]غذاهای غیرقابل‌هضمی که باعث تحریک رشد ریزاندامگان‌های پس‌روده/ کولون می‌شوند.
- Symbiotic [۳]مکمل غذایی حاوی مواد زیست‌یار و کمک‌زیست‌یار.

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Effects of the Glucose Addition during Lactic Fermentation of Rice, Oat and Wheat Flours

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Abstract

Background and objective: Consumer interests in probiotic foods have increased in recent decades. Food industries respond to these growing interests by developing innovative products and guaranteeing high production efficiency. Cereals, due to their prebiotic nature, are good fermentable substrates; from which, potentially functional foods could be achieved. The aim of this study was to verify effects of D-glucose addition on fermentation of rice, oat and wheat flours.

Material and methods: Suspensions of 15% of cereals flours (rice, oat and wheat) in distilled water added with increasing glucose concentrations (2, 5, 7 and 10% w v^{-1}) were fermented by *Lactobacillus paracasei* CBA L74 for 24 h. Then, pH, microbial growth and lactic acid production were assessed.

Results and conclusion: Rice fermentation was not affected by glucose addition. For oat and wheat, addition of D-glucose increased bacterial concentration, as well as lactic acid production. In particular, the best growth was achieved by the addition of 2 and 5% of glucose. Furthermore, lactic acid concentration increased with increased glucose concentration. In conclusion, D-glucose addition seems to be unnecessary for the improvement of rice fermentation. On the contrary, oat and wheat fermentations need further available carbon sources for a better Lactobacillus growth and a higher lactic acid production.

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1. Introduction

Functional foods or foods with additional functions (e.g. health-promotion and disease prevention) have become a new challenge for the food industries. A particular class of functional foods is represented by the probiotics. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" [1]. Based on the literature, the number of lactic fermenting bacteria necessary to temporary colonize the intestine is at least 10^7 CFU g^{-1} [2]. Despite the fact that the most well-known functional foods are dairy products [3], nondairy products such as cereals may contain probiotics [4-6], demostrating their functional activities in several In vitro and In vivo experiment. Cereal flours (rice, oat and wheat) fermented with *Lactobacillus* (*L.*) *paracasei* CBA L-74 have shown abilities to decrease gliadin peptide entrance in Caco-2 cells and promote innate immunity peptides [7-9]. Similarly, a clinical trial has demonstrated the ability of rice flour fermented with a similar bacterial strain in prevention of common infectious diseases in children [10].

The aqueous suspensions of cereal flours are good examples of fermentable matrices since they are rich sources of interesting nutrients such as resistant starch, water-soluble and insoluble β-glucans and arabinoxylans.

These nutrients can help microbial growth as prebiotics, which can selectively stimulate growth and/or activity of a limited number of resident bacterial species in colon and hence improve the host health [11]. A previous study [12] has demonstrated that fermentation of three various water/cereal (rice, oat and wheat) suspensions with a solid content of 15% is possible. In fact, a value considered as sufficient for potentially functional foods was achieved at the end of the fermentation. However, rice flour seemed the best substrate for the microbial growth and lactic acid production. Based on these results, a laboratory-scale experimental study was carried out to understand if glucose addition could improve performance of the fermentation in microbial growth and lactic acid production. Increase of glucose concentration from 0 to 10% w v^{-1} was used in this experiment. The aim of this study was to assess effects of lactic fermentation on cereal flour and potential differences between different flours as well as the potential role of D-glucose on the process.

2. Materials and methods

2.1 Bacterial strain

The bacterial strain used as starter culture was *L. paracasei* CBA L74, (Heinz Italia SpA) with International Depository Accession Number of LMG P-24778. This strain was a Gram-positive homo-fermentative, facultative anaerobic bacteria. The bacteria was stored in freeze-dried form at-20°C and recovered at 37°C, 10 min before the fermentation process, using 0.9% sodium chloride solution. The inoculum bacterial density was 10^8 CFU ml^{-1}.

2.2 Rice, oat and wheat flours

Rice, oat and wheat flours were provided by Heinz Italia SpA. Before fermentation processes, each flour was subjected to heat treatment (120°C for 90 min) to decrease possible microbial loads.

2.3 Laboratory-scale bioreactor for fermentation

The experimental laboratory-scale bioreactor included four parts of a vessel, a mixing system, a system of thermal conditioning and a system for pH and temperature measurements. The vessel was a cylindrical Pyrex glass with an external jacket, allowing circulation of the service fluid. This was necessary to preserve constant temperature of the system. The inner height and diameter respectively included 18 and 10 cm and the external dimensions included 21 and 12 cm, respectively. The vessel maximum capacity was 1.5 L. The mixing system consisted of a stainless steel impeller with a pitched blade turbine and a Rushton turbine blade connected to a motor (a three-phase asynchronous electric motor with 0.25 hp/0.18 kW; 1310 rpm with a speed reducer; 170-880 rpm) that allowed the adjustment of stirring speed. Turbines allowed the axial and radial flows. Therefore, efficient mixing and good homogeneity of the substrates were achieved using a mixing speed of 180 rpm. This was demonstrated by preliminary mixing tests with a food dye (data not shown). Briefly, the best mixing speed (currently 180 rpm) was considered a speed, which allowed perfect distribution of the food dye in suspensions within 10 min. The vessel, the mixing system and the entire mechanism are respectively shown in Figures 1A, B and C. The service fluid used for controlling the fermentation temperature included distilled water (D.W.), thermally regulated using thermo-stated bath, (HAAKE G, USA) at 37°C. A Mettler Toledo probe was used (In Pro 3100, Mettler Toledo, USA) for the pH and temperature measurements.

Figure 1. The vessel and the mixing system. A) The vessel was made of Pyrex glass. It included a jacket vessel; in which, the thermo-stated water was circulated; B) The stainless steel impeller with two various types of turbines: a blade turbine to achieve axial flow and a Rushton turbine to achieve radial flow; and C) The complete equipment used for the laboratory-scale experiments.

2.4 Laboratory-scale fermentation protocol

The fermentation protocol was originally described by Gallo et al. [12] with the exception of added glucose. Briefly, a submerged fermentation under aerobic conditions was carried out in a 1.5-L fermenter with 1-L working volume. First, fermenter and impeller were sterilized at 121°C for 20 min using autoclave. Then, 150 g of each flour (rice, oat or wheat flour) were treated using

dry heat at 120°C for 90 min. Treated flour was mixed with 0.850 L of sterilized D.W. with addition of increasing glucose concentration (0, 2, 5, 7 and 10% w v^{-1} of D-glucose) (Oxoid, UK). This was subjected to a tyndallisation process with two consecutive cycles of heating (90°C) and cooling (37°C) to guarantee the decrease of contaminants [12,13]. During the following fermentation process, temperature of the substrate was preserved at 37°C and the fermentation process was stopped after 24 h. A similar protocol was used for the three flours.

2.5 Analytical methods

Samples were aseptically collected from the bioreactor at various fermentation times (t_0, t_4, t_{14}, t_{20}, t_{24} after the inoculum phase). These samples were used for the pH measurement and microbiological and chemical analyses. Samples were cultured on MRS agar (Oxoid, UK) Petri dishes after serial dilutions and incubated at 37°C for 48 h under anaerobic conditions using special anaerobic kits (Anaerogen Compact, Oxoid, UK). To exclude the contaminants, samples were cultured on Petri dishes prepared with PCA (bacteriological agar, yeast extract, peptose peptone and D-glucose) (Oxoid, USA), MacConkey agar (Oxoid, USA) and gelatine peptone agar (Biolife, Italy). The pH analysis was carried out using In Pro 3100 Probe (Mettler Toledo, USA). Lactic acid concentration was assessed using high performance liquid chromatography (HPLC) (Agilent Technologies 1100, USA) equipped with a C18 Column (Agilent Zorbax C18 4.6 × 150 mm, pore size of 80 A) and a visible/UV detector. The eluent was 0.1 M $NH_4H_2PO_4$ at a flow rate of 0.8 ml min^{-1} and the mobile phase was ammonium phosphate with pH of 2.7 with detection at 218 nm. The analysis temperature was 30°C. Secondary acids such as butyric, acetic and propionic acids were assessed using gas chromatography (Agilent Technologies 6890, USA) equipped with a capillary Poraplot Q Column (25 m × 0.32 mm). The flow rate was 200 ml min^{-1} and the mobile phase was helium gas.

2.6 Statistical analysis

Each experiment was carried out in triplicate. Statistical analysis was carried out using GraphPad Prism Software v.7.0a (San Diego, CA, USA). Mean and standard deviation of the results were calculated and their significance was assessed by Student's t-test. Results were reported as significant when $P \leq 0.05$.

3. Results and discussion

Although dairy foods are the most common substrates used for probiotic production, cereal based foods offer valuable alternatives. Therefore, three various flours (rice, oat and wheat flours) were fermented. The *L. paracasei* CBA L74 was used a fermenting culture. This strain was a homo-fermentative bacterium, meaning that the product is only lactic acid. To verify the success of flour fermentation, lactic acid concentration, pH value and *L. paracasei* growth were assessed.

3.1 Rice flour with increasing glucose concentration

The pH value, bacterial growth and lactic acid production from rice flour are shown in Figure 2 and Table 1. Comparison of results between the rice fermentations with no glucose and with 2% of glucose showed no significant differences in pH value, microbial growth and lactic acid production, as shown in Figures 2A, 2B and 2C, respectively. In both conditions (with no glucose and with 2% of glucose), the statistically significant differences were recorded at t_4 ($P<0.05$), t_{14} ($P<0.01$) and t_{20} ($P<0.05$) regarding bacterial growth and at t_{14} ($P<0.01$) and t_{20} ($P<0.05$) regarding lactic acid production. However, no differences were recorded at the end of the process (t_{24} pH value of 3.42 ±0.044 with no added glucose and 3.42 ±0.190 with 2% of added glucose; t_{24} bacterial growth of 9.5×10^8 CFU ml^{-1} $\pm 1.33 \times 10^8$ with no added glucose and 1×10^9 CFU ml^{-1} $\pm 2.57 \times 10^8$ with 2% of added glucose, starting with an initial bacterial load of nearly 5×10^6 CFU ml^{-1}; lactic acid production of 3100 mg l^{-1} ±134.55 with no added glucose and 3200 mg l^{-1} ±94.79 with 2% of added glucose). Moreover, presence of the secondary acids (acetic, propionic and butyric acids) was assessed. No significant quantities of these acids were detected (data not shown), demonstrating absence of the contaminants. Considering no differences between the fermentations with no added glucose and that with 2% of added glucose, no other experiments with increasing glucose concentrations were reported as useful.

Table 1. Summary of the results from the rice fermentation without or with 2% of added glucose

		Rice	
	Time (h)	0%	2%
pH	0	6.32 ± 0.14	6.04 ± 0.26
	24	3.42 ± 0.04	3.42 ± 0.19
Growth (CFU ml^{-1})	0	$5.0\times10^6 \pm 6.0\times10^5$	$6.0\times10^6 \pm 5.5\times10^5$
	24	$9.5\times10^8 \pm 1.33\times10^8$	$1.0\times10^9 \pm 2.57\times10^8$
Lactic Acid (mg l^{-1})	0	0	0
	24	3100 ±134.55	3200 ± 94.80

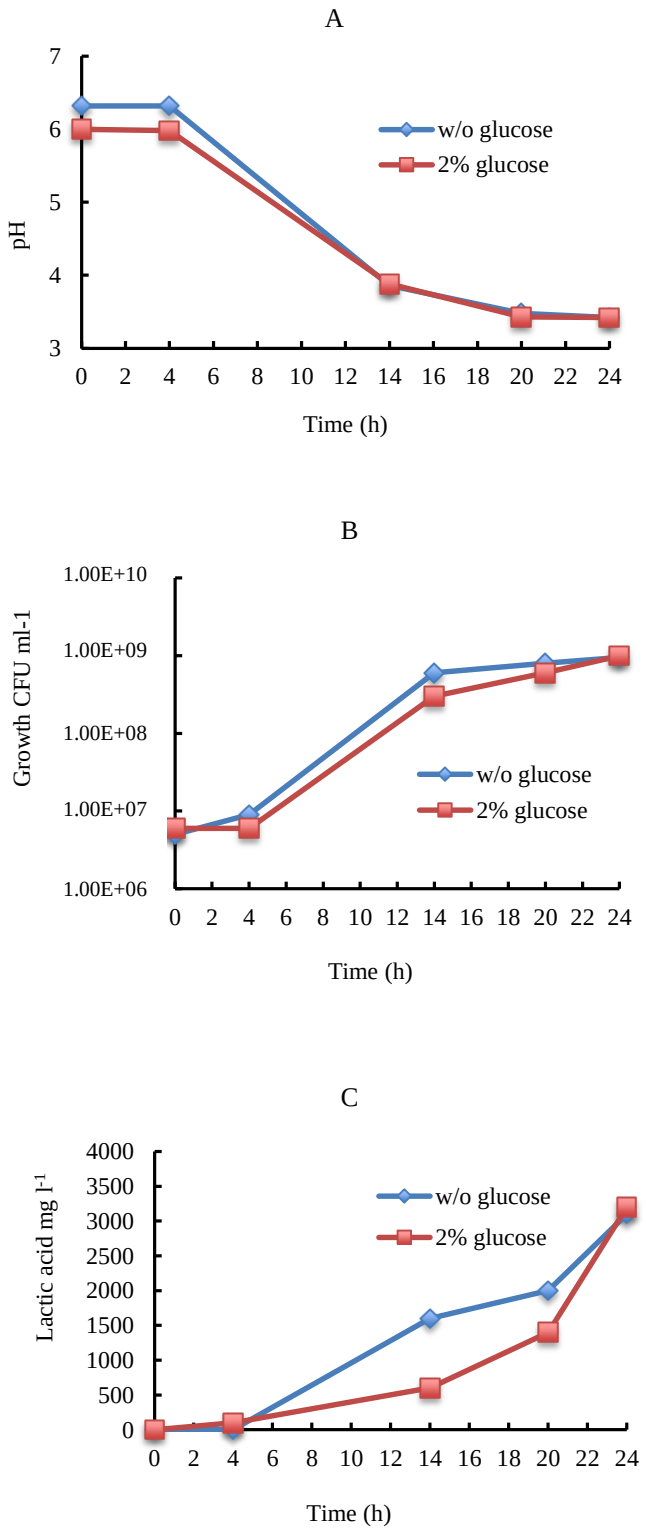

Figure 2. Analytical results from fermentation of the rice. A) The pH values of the rice fermentation with no glucose addition and with 2% of added glucose; B) The bacterial load (CFU ml^{-1}) of the rice fermentation with no glucose addition and with 2% of added glucose; and C) The lactic acid production (mg l^{-1}) during rice fermentation with no glucose addition and with 2% of added glucose. Each result was the mean value of a triplicate analysis, Student t-test; *P<0.05; **P<0.01

3.2 Oat flour with increasing glucose concentration

The pH value, bacterial growth and lactic acid production from oat flour are shown in Figure 3 and Table 2. Comparison of results from oats with no added glucose and with 2% of added glucose showed multiple differences, which led to test glucose concentrations greater than 2%. To assess effects of glucose addition (5-10%), bacterial growth and lactic acid concentration were assessed at t_0 and t_{24} and the values were compared with the values of 0 and 2% of glucose. The pH results are demonstrated in Figure 3a. All fermentations included an initial pH of nearly 6.2.

At t_{24}, oat fermentations with 0, 2 and 5% of added glucose reached similar pH values (0%, 4.51 ±0.11; 2%, 4.51 ±0.097 and 5%, 4.56 ±0.015), while fermentations with 7 and 10% of added glucose reached a lower value (7%, 3.84 ±0.026 and 10%, 3.84 ±0.067). Differences between the fermentations with lower glucose concentrations (0, 2 and 5%) and those with higher glucose concentrations (7 and 10%) were statistically significant (P<0.001) (Figure 3B).

Results of the microbial growth are shown in Figure 3C. Significant differences were seen at t_{24} between the no added glucose condition and all other conditions (0%, 2.00 × 10^8 CFU ml^{-1} ±3.79 × 10^7; 2%, 5.47 × 10^9 CFU ml^{-1} ±5.86 × 10^8; 5%, 8.1 × 10^9 CFU ml^{-1} ±4.36 × 10^8; 7%, 1.29 × 10^9 CFU ml^{-1} ±4.52 × 10^8 and 10%, 1.53 × 10^9 CFU ml^{-1} ±6.2 × 10^8). Statistically significant differences were reported for all the results, except for the difference between the fermentations with 7 and 10% of glucose (Figure 3D). Interestingly, a higher bacterial load was reached at t_{24} by adding 5% of glucose. For the concentrations of glucose greater than 5%, the final bacterial load decreased significantly. However, this load was still higher than that observed when no glucose was added. Therefore, oat fermentation possibly needs addition of glucose. However, glucose concentrations greater than 5% result in substrate inhibition, at least when bacterial growth is concerned. Results of the lactic acid production are shown in Figure 3E. As the glucose concentration increased; lactic acid production increased with statistically significant differences (Figure 3F). Starting all conditions with the absence at t_0 of lactic acid, they reached t_{24} 2000 mg l^{-1} ±94.98 when no glucose was added and 3300 mg l^{-1} ±132.64, 3850 mg l^{-1} ±147.42, 4500 mg l^{-1} ±196.22 and 5800 mg l^{-1} ±314.22 when 2, 5, 7 and 10% of glucose were respectively added. This could explain the lowest pH levels observed at t_{24}, when 7 and 10% of glucose were added to the oat. In this study, presence of the secondary acids (acetic, propionic and butyric acids) was assessed. No significant quantities of these acids were seen (data not shown), proving absence of the contaminants.

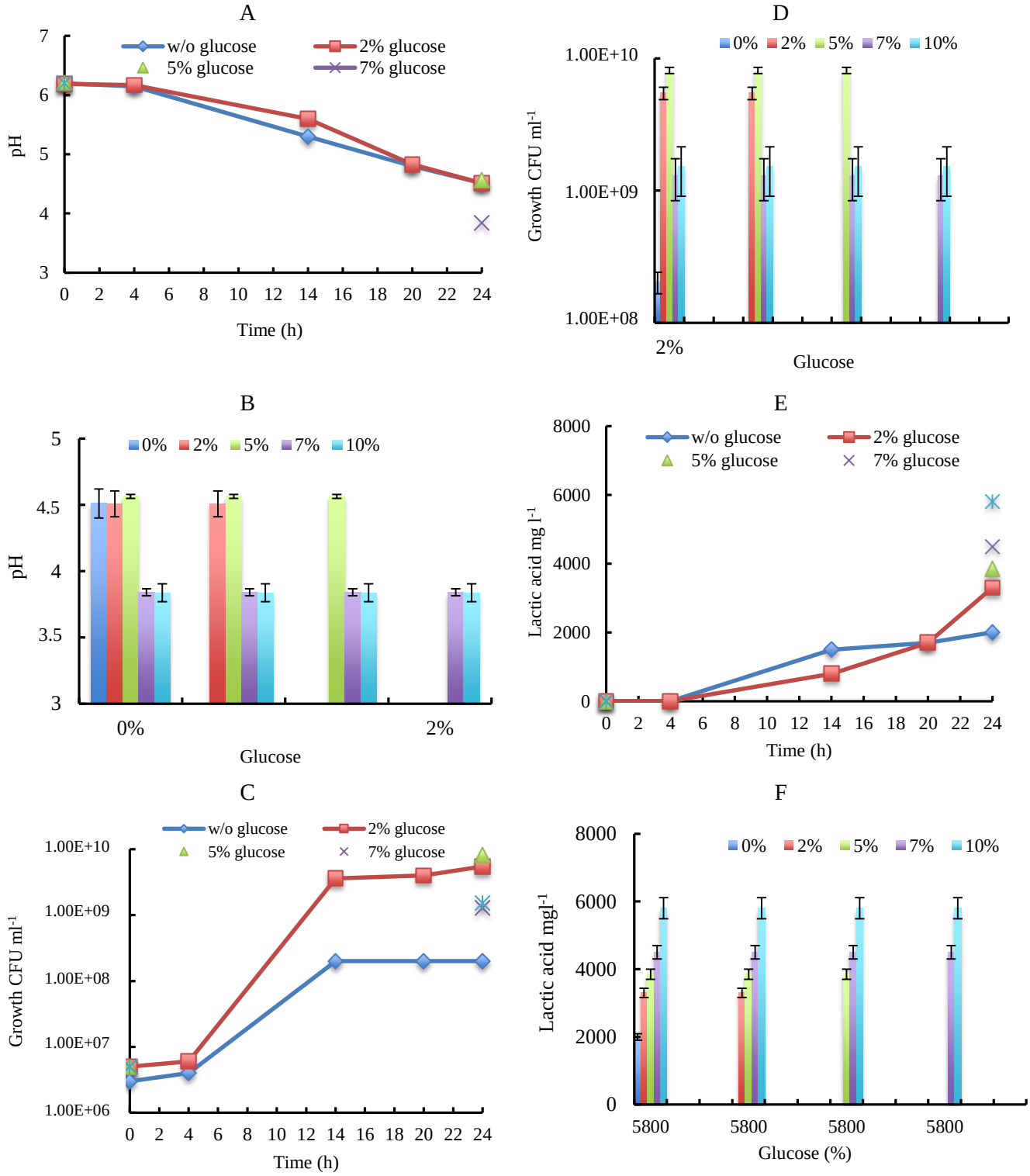

Figure 3. Analytical results of the fermentation of oat flour with increasing added glucose concentration. A) The pH values of the oat fermentation with 0, 2 (t_0, t_4, t_{14}, t_{20}, t_{24}), 5, 7 and 10% (t_0, t_{24}); B) Statistical results by the comparison between t_{24} pH values of the glucose concentrations; C) Bacterial load (t_0) and growth of the oat flour fermented with 0, 2 (t_0, t_4, t_{14}, t_{20}, t_{24}), 5, 7 and 10% (t_0, t_{24}) of glucose; D) Statistical results by the comparison of t_{24} bacterial load achieved using each glucose concentration with t_{24} bacterial load achieved using other glucose concentrations; E) Lactic acid production (mg l^{-1}) in the oat flour fermented with 0, 2 (t_0, t_4, t_{14}, t_{20}, t_{24}), 5, 7 and 10% (t_0, t_{24}) of glucose; and F) Statistical results by the comparison of t_{24} lactic acid produced using each glucose concentration with t_{24} lactic acid produced using other glucose concentrations. To make a statistically significant experimental campaign, each experiment was carried out in triplicate, Student t-test; *p< 0.05; **P<0.01; ***P<0.001

Table 2. Summary of the results from the oat fermentation with increasing glucose addition

		Oat				
	Time (h)	0%	2%	5%	7%	10%
pH	0	6.20 ± 0.04	6.19 ± 0.025	6.20 ± 0.021	6.20 ±0.036	6.20 ± 0.032
	24	4.51 ± 0.11	4.51 ± 0.097	4.56 ± 0.015	3.84 ± 0.026	3.84 ± 0.067
Growth (CFU ml^{-1})	0	$3.0\times10^6 \pm 7.4\times10^5$	$5.0\times10^6 \pm 3.8\times10^5$	$5.0\times10^6 \pm 3.6\times10^5$	$5.0\times10^6 \pm 3.1\times10^5$	$5.0\times10^6 \pm 3.2\times10^5$
	24	$2.0\times10^8 \pm 3.8\times10^7$	$5.5\times10^9 \pm 5.86\times10^8$	$8.1\times10^9 \pm 4.4\times10^8$	$1.3\times10^9 \pm 4.5\times10^8$	$1.5\times10^9 \pm 6.2\times10^8$
Lactic Acid (mg l^{-1})	0	0	0	0	0	0
	24	2000 ± 94.98	3300 ± 132.64	3850 ± 147.42	4500 ± 196.22	5800 ± 314.22

Differences with no statistical significance were recorded when glucose was added (Figure 4D), verifying the use of glucose for a better microbial growth in wheat fermentation. No statistically significant differences were seen between 7 and 10% of added glucose. Similar to oat, a higher t_{24} bacterial load was reached by adding 5% of glucose. For glucose concentrations greater than 5%, the final bacterial load significantly decreased. The lactic acid production results are shown in Figure 4E. The initial lactic acid concentration was 0 mg l^{-1}. At t_{24}, production of 2300 mg l^{-1} ±174.51 was achieved when no glucose added and 3500 mg l^{-1} ±116.66, 5850 mg l^{-1} ±94.18, 5860 mg L^{-1} ±32.88 and 8000 mg l^{-1} ±133.69 when 2, 5, 7 and 10% of glucose were used, respectively. As glucose concentration increased, lactic acid production increased with statistically significant differences between the increasing concentrations, except between fermentations with 5 and 7% of glucose (Figure 4F). Presence of the secondary acids (acetic, propionic and butyric acids) was assessed. No significant quantities of these acids were seen (data not shown), demonstrating absence of the contaminants.

3.3 Wheat flour with increasing glucose concentration

The pH values, bacterial growth and lactic acid production with wheat flour are shown in Figure 4 and Table 3. Comparison of results from wheat with no added glucose and with 2% of added glucose showed several differences, which led to test concentrations of glucose greater than 2%. To assess effects of glucose addition (5-10%), bacterial growth and lactic acid concentration were assessed at t_0 and t_{24}. These values were compared to those with 0 and 2% of glucose. The pH results are shown in Figure 4A. For all conditions, pH value at t_0 included nearly 6.2. Furthermore, significant differences were seen at t_{24} between the fermentation with no glucose addition and all other conditions (pH values at t_{24} included 4.1 ±0.1 for 0%; 3.26 ±0.27 for 2%; 3.3 ±0.27 for 5%; 3.20 ±0.21 for 7% and 3.5 ±0.2 for 10% of the added glucose) (Figure 4B).

Results of the microbial growth are demonstrated in Figure 4C. In general, the bacterial load for all conditions 5×10^6 CFU ml^{-1}. The bacterial load at t_{24} included 3×10^8 CFU ml^{-1} $\pm 4.58 \times 10^7$ when no glucose was added and 4.5×10^9 CFU ml^{-1} $\pm 4.36 \times 10^8$ for 2%, 8×10^9 CFU ml^{-1} $\pm 4.51 \times 10^8$ for 5%, 2×10^9 $\pm 3.22 \times 10^8$ for 7% and 1.53×10^9 CFU ml^{-1} $\pm 5.13 \times 10^8$ for 10% of added glucose, respectively.

Table 3. Summary of the results from the wheat fermentation with increasing glucose addition

		Wheat				
	Time (h)	0%	2%	5%	7%	10%
pH	0	6.0 ± 0.10	6.0 ± 0.10	6.2 ± 0.07	6.2 ± 0.08	6.2 ± 0.05
	24	4.10 ± 0.10	3.26 ± 0.27	3.3 ± 0.27	3.20 ± 0.21	3.5 ± 0.2
Growth	0	$6.0\times10^6 \pm 9.3\times10^5$	$4.4\times10^6 \pm 4.1\times10^5$	$5.1\times10^6 \pm 6.6\times10^5$	$5.1\times10^6 \pm 1.0\times10^6$	$5.1\times10^6 \pm 6.7\times10^5$
(CFU ml^{-1})	24	$3.0\times10^8 \pm 4.6\times10^7$	$4.5\times10^9 \pm 4.4\times10^8$	$8.0\times10^9 \pm 4.5\times10^8$	$2.0\times10^9 \pm 3.2\times10^8$	$1.5\times10^9 \pm 5.1\times10^8$
Lactic Acid	0	0	0	0	0	0
(mg l^{-1})	24	2300 ± 174.51	3500 ± 116.66	5850 ± 94.18	5860 ± 32.88	8000 ± 133.69

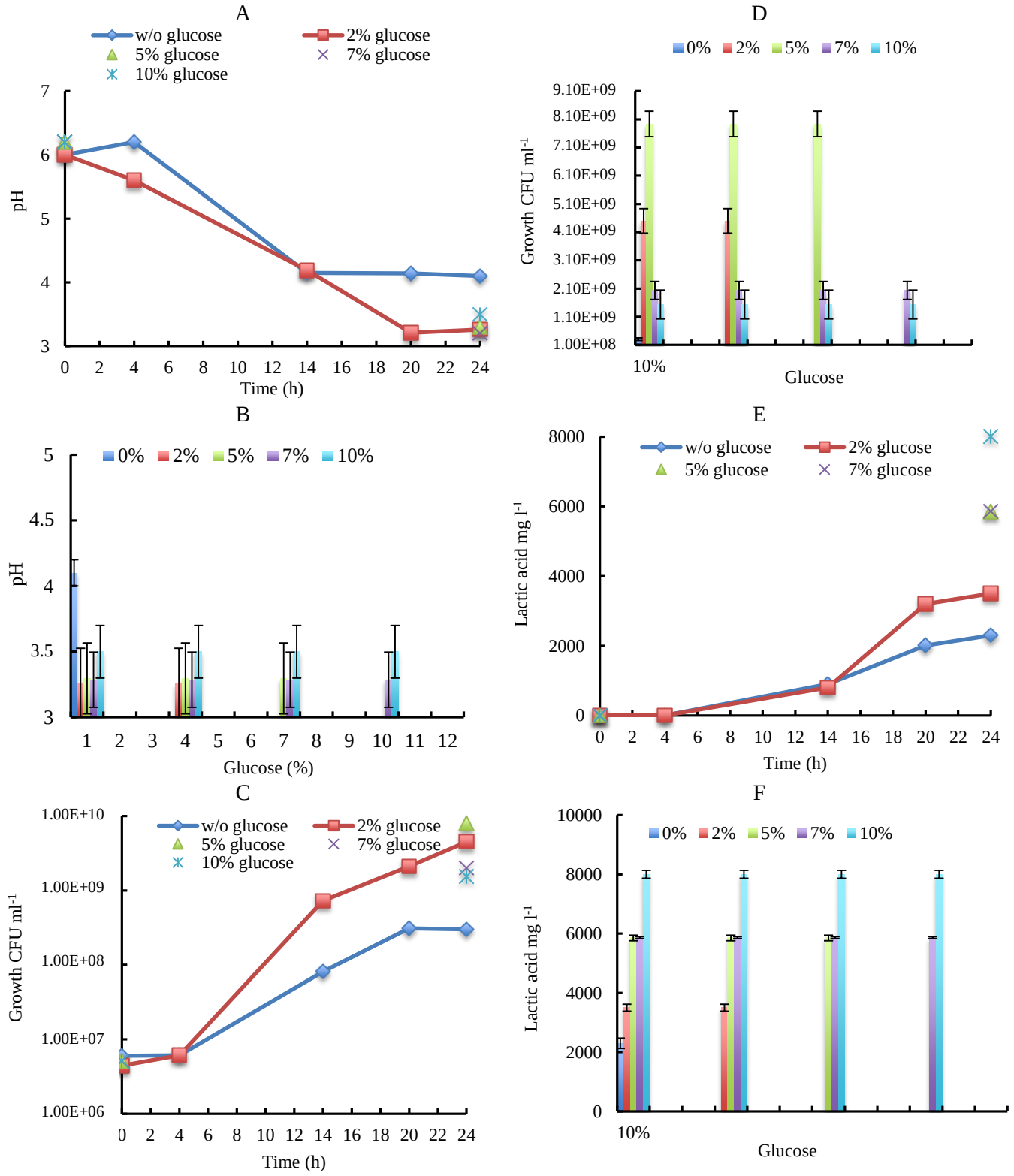

Figure 4. Analytical results of the fermentations of wheat flour with increasing added glucose concentration. A) The pH values of the wheat fermentation with 0, 2 (t_0, t_4, t_{14}, t_{20}, t_{24}), 5, 7 and 10% (t_0, t_{24}) of glucose; B) Statistical results by the comparison of t_{24} pH value using each glucose concentration with t_{24} pH value using other glucose concentrations; C) Bacterial load (t_0) and growth in wheat fermented with 0, 2 (t_0, t_4, t_{14}, t_{20}, t_{24}), 5, 7 and 10% (t_0, t_{24}) of glucose; D) Statistical results by the comparison of t_{24} bacterial load achieved using each glucose concentration with t_{24} bacterial load achieved using other glucose concentrations; E) Lactic acid production (mg l^{-1}) of the wheat flour fermented with 0, 2 (t_0, t_4, t_{14}, t_{20}, t_{24}), 5, 7 and 10% (t_0, t_{24}) of glucose; and F) Statistical results by the comparison of t_{24} lactic acid produced using each glucose concentration with t_{24} lactic acid produced using other glucose concentrations. Each experiment was carried out in triplicate, Student t-test; *P<0.05; **P<0.01; ***P<0.001

3.4 Comparison between rice, oat and wheat fermentations with increasing glucose concentration

Comparison of the results from experiments with no glucose addition to rice, oat and wheat flours with the results from the addition of 2% w v^{-1} of glucose showed an improved process for the oat and wheat flours but not for the rice. Therefore, experiments continued on oat and wheat flours, investigating increased concentration of D-glucose (5, 7 and 10%). In this preliminary study, only two sampling times of t_0 and t_{24}were used. By the addition of 2% of D-glucose, Lactobacillus growth on the oat and wheat flours was a log higher, compared to that with no glucose addition. This was not observed at the end of the rice flour fermentation. The lack of bacterial growth promotion could be due to several factors. First, rice starch granules include the smallest size [14,15] and, as suggested by Bhatty and Vasanthan [16], small granules are hydrolyzed more rapidly than large granules by α-amylase, which could make rice starch more accessible than oat and wheat starch to hydrolysis. Second, high amylose starches are particularly resistant to hydrolysis [17] and rice seems to include a smaller amylose content (nearly 21-25% w w^{-1} on a total starch dry basis) [18] than that oat (nearly 27.5-29.8% w w^{-1} on a total starch dry basis) [19] and wheat (nearly 25.6% w w^{-1} on total starch dry basis) [18] do. These data of granule size and amylose content could explain easier hydrolysis of the rice starch. Another factor that facilitates greater accessibility of the rice starch could be linked to its crystalline type. The rice starch is composed of an A structure, while oat and wheat starches are composed of two A and B types of the crystal structures. It has been reported [16,20] that type B starches resist enzymatic hydrolysis, compared to that type A starches do. This could explain why no differences were seen with or without glucose addition at the end of the rice fermentation in the current study. In contrast, the higher difficulty in hydrolyzing oat and wheat granules was possibly linked to D-glucose. For the lactic acid production at the end of the fermentations with no glucose addition, the highest concentration was reported in rice flour (3100 mg l^{-1}), while significantly lower concentrations were reported in oat (2000 mg l^{-1}) and wheat (2300 mg l^{-1}) flours. By the addition of 2% w v^{-1} of D-glucose, results changed drastically since lactic acid production increased to 3300 mg l^{-1} for oat and to 3500 mg l^{-1} for wheat flours. However, no similar results were seen in the rice flour (3200 mg l^{-1}). This was possibly due to the growth of Lactobacillus. Assessing effects of increased glucose concentration of D-glucose (0-10%) on oat and wheat flours demonstrated improved bacterial growth. within a certain threshold for oat and wheat flours, the greatest growth was achieved with the addition of 2 and 5% of glucose. However, addition of higher quantities resulted in substrate inhibition. For lactic acid production in oat and wheat flours, increases in the added glucose increased the lactic acid production. It is well-known that glucose is the major substrate to improve lactic fermentation. The unusual finding based on the present study is the improved process in terms of pH value, bacterial growth and lactic acid production in wheat and oat flours with various glucose concentrations. Calderon [21,22] carried out a similar study, using potato starch alone and potato starch with glucose. He reported increased bacterial growth of *L. fermentum* Ogi E1 and doubled lactic acid production when glucose was added to the starch, as reported from the present study on oat and wheat flours.

4. Conclusion

In the current study, lactic fermentation of cereal flours was possible but results were different in terms of strain growth and lactic acid production. To achieve the best results for oat and wheat fermentations, it is preferable to add D-glucose to the flour. Furthermore, the sugar concentration depends on the target; therefore, using a maximum concentration of 5% D-glucose is favorable for a better bacterial growth while a glucose concentration higher than 5% can be used for a higher lactic acid production. However, rice fermentation does not need this sugar additive.

Contributions: Roberto Nigro and Marianna Gallo designed the research; Rosa Colucci Cante, Federica Nigro, Francesca Passannanti, Dana Salameh, Paola Schiattarella and Concetta Schioppa carried out the research; Andrea Budelli provided the raw materials and *Lactobacillus paracasei* CBA L74; Marianna Gallo and Francesca Passannanti wrote the paper and have the primary responsibility for the final content. All authors have read and approved the final manuscript.

5. Acknowledgements

Dr. Andrea Budelli is currently employed by Heinz BV, Netherlands. He provided the raw materials (rice, oat and wheat flours) and *Lactobacillus paracasei* CBA L74 and participated in design of the study. He had no additional role in data collection and analysis, decision to publish or preparation of the manuscript. Heinz BV did not provide any financial supports to the authors for experimental activities and did not have additional roles in study design, data collection and analysis, decision to publish or preparation of the manuscript.

6. Conflict of interest

The authors declare no conflict of interest.

References

1. Health, food and agricultural organization of the United Nations and world. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. World Health Organization, 2001.
2. International dairy federation. Standards for fermented milks. 1997: D-Doc 316.
3. Leatherhead food international. The international market for functional foods, 3rd edition. Functional food market report, 2006.
4. Lamsal BP, Faubion JM. The beneficial use of cereal and cereal components in probiotic foods. Food Rev Int. 2009; 25(2): 103-114. doi: 10.1080/87559120802682573
5. Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JAF. Functional foods and nondairy probiotic food development: Trends, concepts, and products. Compr Rev Food Sci Food Saf. 2010: 9:292-302. doi: 10.1111/j.1541-4337.2010.00110
6. Prado FC, Parada JL, Pandey A, Soccol CR. Trends in non-dairy probiotic beverages Food Res Int. 2008: 41:111-123. doi: 10.1007/s13197-015-1795-2
7. Sarno M, Lania G, Cuomo M, Nigro F, Passannanti F, Budelli A, Fasano F, Troncone R, Auricchio S, Barone MV, Nigro R, Nanayakkara M. *Lactobacillus paracasei* CBA L74 interferes with gliadin peptides entrance in Caco-2 cells. Int J Food Sci Nutr. 2014; 65(8): 953-959. doi: 10.3109/09637486.2014.940283.
8. Gallo M, Nigro F, Passannanti F, Nanayakkara M, Lania G, Parisi F, Salameh D, Budelli A, Barone MV, Nigro R. Effect of pH control during rice fermentation in preventing a gliadin p31-43 entrance in epithelial cells. Int J Food Sci Nutr. 2019 ; 10: 1-9. doi: 10.1080/09637486.2019.1599827.
9. Paparo L, Aitoro R, Nocerino R, Maddalena Y, Caprio S, Amato F, Pezzella V, Cosenza L, Budelli A, Fasano F, Di Scala C, Berni Canani R. Effects of direct interaction between fermented milk and rice novel dietary products with human enterocytes on cell growth and innate immunity peptides production. Dig Liver Dis. 2014; 46: e97. doi: 10.1016/j.dld.2014.07.085
10. Nocerino R, Paparo L, Terrin G, Pezzella V, Amoroso A, Cosenza L, Cecere G, De Marco G, Micillo M, Albano F, Nugnes R, Ferri P, Ciccarelli G, Giaccio G, Spadaro R, Maddalena Y, Berni Canani F, Berni Canani R. Cow's milk and rice fermented with *Lactobacillus paracasei* CBA L74 prevent infectious diseases in children: A randomized controlled trial. Clin Nutr. 2017; 36(1): 118-125 doi: 10.1016/j.clnu.2015.12.004
11. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J Nutr. 1995: 125(6): 140-1412. doi: 10.1093/jn/125.6.1401
12. Gallo M, Nigro F, Passannanti F, Salameh D, Schiattarella P, Budelli A, Nigro R. Lactic fermentation of cereal flour: feasibility tests on rice, oat and wheat. Appl Food Biotechnol. 2019, 6 (3): 165-172. doi: 10.22037/afb.v4i3.15014
13. Gallo M, Nigro F, Passannanti F, Salameh D, Budelli A, Marzocchella A, Nigro R. rice fermentation by *Lactobacillus paracasei* CBA L74. Int J Rice Res. 2018; 103. doi: 10.29011/IJRR-103. 000003
14. Tester RF, Qi X, Karkalas J. Hydrolysis of native starches with amylases. Anim Feed SciTechnol. 2006; 130: 39-54. doi: 10.1016/j.anifeedsci.2006.01.016
15. Aller E, Abete I, Astrup A, Martinez JA, van Baak MA. Starches, sugars and obesity. Nutrients 2011; 3: 341-369. doi: 10.3390/nu3030341
16. Bhatty T, Vasanthan RS. Physicochemical properties of small-and large-granule starches of waxy, regular and high-amylose barleys. Cereal Chem. 1996: 73: 199-207. doi: 10.1016/S0008-6215(00)00292-5
17. Gallant D, Bouchet B, Buleon A, Perez S. Physical characteristics of starch granules and susceptibility to enzymatic degradation. Eur J Clin Nutr. 1992: 46: S3-S16.
18. Alcazar-Alay SC, Almeida Meireles MA. Physicochemical properties, modifications and applications of starches from different botanical sources. Food Sci Technol Campinas. 2015: 35(2): 215-236. doi: 10.1590/1678-457X.6749
19. Tester RF, Karkalas J. Swelling and gelatinization of oat starches. Cereal Chem. 1996; 73(2): 271-277.
20. Gerard C, Colonna P, Buleon A, Planchot V. Amylolysis of maize mutant starches. J Sci Food Agric. 2001; 81: 1281-1287. doi: 10.1002/jsfa.929
21. Calderon M, Loiseau G, Rodriguez R, Guyot JP. Study of starch fermentation at low pH by *Lactobacillus fermentum* Ogi E1 reveals uncoupling between growth and a-amylase production at ph 4.0. Int J Food Microbiol. 2003; 80(1):77-87. doi: 10.1016/S0168-1605(02)00140-X
22. Calderon M, Loiseau G, Guyot JP. Fermentation by *Lactobacillus fermentum* Ogi E1 of different combinations of carbohydrates occurring naturally in cereals: consequences on growth energetics and a-amylase production. Int J Food Microbiol. 2003; 80 161-169. doi: 10.1016/S0168-1605(02)00147-2

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اثرات افزودن گلوکز هنگام تخمیر لاکتیکی آردهای برنج، جو دوسر و گندم

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چکیده

سابقه و هدف: علاقه مصرف کنندگان مواد غذایی زیست‌یار[1] در دهه های اخیر افزایش یافته است. صنایع غذایی با تولید فرآورده های نوآورانه و تضمین بهره‌وری بالای تولید، به این توجه فزاینده پاسخ می دهند. غلات، به علت ماهیت کمک زیست‌یاری[2]، رشدمایه هایی[3]قابل تخمیر مناسبی می باشند؛ که امکان تولید مواد غذایی فراسودمند[4] از آن وجود دارد. هدف این مطالعه تایید اثرات افزودن د-گلوکز بر تخمیر آردهای برنج، جو دوسر و گندم بود.

مواد و روش ها: سوسپانسیون ۱۵ درصدی آرد غلات (برنج، جو دوسر و گندم) در آب مقطر با افزایش غلظت گلوکز (۲، ۵، ۷ و ۱۰ وزنی حجمی) به مدت ۲۴ ساعت توسط با *لاکتوباسیلوس پاراکازئی* CBA L74 تخمیر شدند. سپس، pH، رشد میکروبی و میزان تولید لاکتیک اسید بررسی شد.

یافته‌ها و نتیجه‌گیری: تخمیر برنج تحت تاثیر افزودن گلوکز نمی باشد. درمورد جو دوسر و گندم، افزودن د-گلوکز غلظت باکتریایی و نیز تولید لاکتیک را افزایش داد. به خصوص، بیشترین رشد با افزودن ۲ و ۰/۵ درصد گلوکز به دست آمد. علاوه بر این، غلظت لاکتیک اسید با افزایش غلظت گلوکز افزایش یافت. در نتیجه، به نظر می رشد افزودن د-گلوکز برای بهبود تخمیر برنج ضرورتی نداشته باشد. در مقابل، تخمیر جو دوسر و گندم برای رشد بهتر لاکتوباسیلوس و تولید بیشتر لاکتیک اسید، نیازمند دسترسی به منابع کربن بیشتر می باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- تخمیر لاکتیکی
- *لاکتوباسیلوس پاراکازئی* CBA L74
- زیست یار

***نویسنده مسئول**

ماریانا گالو

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[1] Probiotic foods
[2] Prebiotic
[3] Substrate
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ORIGINAL ARTICLE

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Biotechnological production of natural sweeteners and preservatives on tomato paste

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Abstract

There is a growing interest in healthier foods and cleaner labels. In particular, foods containing a lower sugar concentration but with the same organoleptic properties and the same shelf life are becoming a new challenge. To this end, we performed a first set of experimentations on two simple broths (LFM1 and LFM2), which differed in their glucose concentration (250 g/L and 200 g/L, respectively), and a second set of fermentations on two diluted tomato pastes (TP1 and TP2), with the same glucose concentration but different in the presence (TP2) or absence (TP1) of yeast extract. These substrates were fermented by *Yarrowia lipolytica* ATCC 8661 at 30°C for 192 hr, to understand its ability in simultaneous production of erythritol and citric acid. Although the process should be optimized, in all the cases tested we obtained the erythritol and the citric acid production.

Practical application

The aim of the present study was the simultaneous production of erythritol and citric acid on a food matrix. This is a preliminary study to obtain, directly by fermentation, a natural sweetener able to give the food product, in particular tomato paste, a sweet taste without adding artificial sweeteners. At the same time, we tried to produce citric acid as a natural preservative to improve shelf life. In this way, we wish to obtain, through several experimentations, semifinished products for the production of vegetal soups and sauces.

1 | INTRODUCTION

In an increasingly industrialized era, consumers pay more and more attention to the quality of the food, to the ingredients contained in it, and to what is written on the label. For example, consumers demand a higher quality of the product, without using preservatives (Zink, 1997) with a longer shelf life. Since some ingredients (such as artificial additives) are not well accepted by consumers, industries are increasingly driven to look for "more natural" ingredients or innovative processes that guarantee the same organoleptic properties, the same shelf life and the same food safety. This increased need for information by consumers depends on a greater awareness of the relationship between diet and health. Despite this greater attention to clean label products, excessive consumption of sugar is still recorded (Foreign Agricultural Service/USDA, 2017), and it is considered as the main cause of various diseases, including obesity, diabetes, and dental caries (Runnel et al., 2013; Stanhope, 2016). For this reason, the WHO recommends reducing the intake of free sugars to less than 10% of total energy intake (World Health Organization, 2015). Various types of food products can be found on the market in which sucrose is partially or totally replaced by noncaloric or low-caloric sweeteners, of natural or synthetic (artificial) origin, which have a lower caloric content than the common sucrose.

Abbreviations: LFM, liquid fermentation medium; TP, tomato paste.

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Because of the consumers', and thus industries', growing interest in food products characterized by a clean label, the final aim of our work was the simultaneous production of erythritol, a natural sweetener, and citric acid, a natural preservative, through batch fermentation. Erythritol is a four-carbon polyalcohol with a limited caloric intake (0.2 kcal/g), with a glycemic index equal to zero, making it ideal for diabetic patients or for patients affected by impaired glucose tolerance (Wölnerhanssen et al., 2016); furthermore, it also had shown a preventing effect on vascular function in patients with type 2 diabetes (Flint et al., 2013). Its sweetening power has been estimated around 60–80% of the common sucrose; moreover, erythritol does not accumulate in the intestinal lumen, thus limiting the possibility of diarrhea and cramp-like abdominal pains, instead allowing intestinal absorption and consequent elimination by the renal route (Regnat, Mach, & Mach-Aigner, 2018); it does not have the bitter aftertaste typical of other polyalcohols. Erythritol can be produced either by chemical methods (Otey, Sloan, Wilham, & Mehltretter, 1961) or by biotechnological methods, through fermentation by yeasts and bacteria (Moon, Jeya, Kim, & Lee, 2010). As for citric acid, it is a tricarboxylic acid, recognized worldwide as GRAS (generally recognized as safe). The food and pharmaceutical industries use citric acid for its pleasant acid aftertaste, for its strong solubility in water and its chelating properties (Soccol, Vandenberghe, & Rodrigues, 2006). The aim of the work was to test the factors influencing the growth of the yeast used and the production of metabolites such as citric acid and erythritol, during fermentations performed, firstly using culture broth and subsequently on a more complex matrix like the tomato paste (TP). As microorganism *Yarrowia lipolytica* was used; this is a yeast with great potential in food applications (Zinjarde, 2014), defined as safe-to-use (Groenewald et al., 2013) and considered promising in citric acid (Cavallo, Charreau, Cerrutti, & Foresti, 2017) and polyols (Liu, Ji, & Huang, 2015) production.

2 | MATERIALS AND METHODS

In the present work, *Y. lipolytica* was used as fermentative strain. In a first set of tests a culture broth (LFM—liquid fermentation medium) was used as a fermentation substrate containing different nutrients that provide the source of carbon (glucose), the source of nitrogen (yeast extract), and the growth factors (KH_2, PO_4, and $MgSO_4$) for the yeast. The culture broth used had the same composition of the medium used by Savergave (2011) in his work. Later on, the tests were carried out on the same substrate but with a lower glucose concentration (LFM2). Finally, the TP was used as substrate with the addition of the carbon source (glucose and called TP1) and then with the addition of the nitrogen source (yeast extract; TP2). In this way we tested the strain's ability to simultaneously produce erythritol and citric acid, on a simple broth at two different conditions (glucose concentration) and on TP with and without the nitrogen source.

2.1 | Lab-scale reactor

The experimental laboratory plant was composed of four components: a vessel, a mixing system, a system of thermal conditioning, and a system for pH and temperature measurements. The vessel, with a maximum capacity of 1.5 L, was cylindrical Pyrex (20 cm high, 10 cm ID) (Glass Studio Napoli, NA, Italy) equipped with an external jacket that allowed the circulation of a service fluid necessary to maintain the entire apparatus at a constant temperature. The fluid was thermoregulated through a thermostated bath (HAAKE G) (Haake srl, Rezzato BS, Italy), set at 30°C. The mixing system consisted of a stainless steel impeller, having an inclined blade turbine and a Rushton turbine, connected to a motor that allowed adjustment of the stirring speed. The first turbine allowed axial flow while the second a radial flow. In this way a perfect mixing and, therefore, homogeneity were ensured, as demonstrated by preliminary mixing tests conducted with a food dye (not shown). As system for pH and temperature measurement, a Mettler Toledo probe was used (In Pro 3100) (Mettler-Toledo S.p.A., Novate Milanese MI, Italy).

2.2 | Microorganism

The choice of microorganism was made with the premise of using a strain that is not mutagenized.

Y. lipolytica ATCC 8661 (NCIM 3472) was procured from American type culture collection (ATCC). That strain was a forced aerobic; its optimal growth temperature was 24°C and the optimal medium was yeast mold (YM). *Y. lipolytica* was stored at −20°C in YM broth (BD 271120) enriched with glycerol 10%, and revitalized in fresh broth at 25°C for 48 hr. After that time of revitalization, the preinoculum was added to the fermenting media, described below, to begin fermentation.

2.3 | Fermentation media

The process of erythritol and citric acid production was tested on four different media: LFM1, LFM2, TP1, and TP2. The LFM1 medium was composed of glucose (Romil) 250 g/L, yeast extract (Sigma-Aldrich) 10 g/L, KH_2PO_4 (Carlo Erba) 5 g/L, $MgSO_4$ (Carlo Erba) 0.25 g/L, in distilled water. The LFM2 medium had the same composition of the LFM1 medium, the only difference being the glucose concentration (200 g/L).

The following set of experimental tests was carried out using TP (brand Cirio) as substrate, adding the carbon source (glucose) and the nitrogen source (yeast extract). TP1 was composed of 420 g of TP, 420 g of distilled water, and 199 g of glucose. The glucose was added to obtain a total glucose concentration of 250 g/L. Afterwards 10 g/L of yeast extract was added to study the effect of a higher nitrogen source. Therefore, the composition of TP2 was 420 g of TP, 420 g of distilled water, 199 g of glucose, and 7.96 g of yeast extract.

2.4 | Fermentation protocol

Fermentation tests were performed ensuring that the whole process was carried out under sterile conditions: every device was previously sterilized, the reactor was assembled and charged under laminar flow hood and sampling was done in the same way; furthermore, the reactor ensured a perfect seal. All batch cultures were performed in a 1 L fermenter at 30°C for 192 hr, with a working volume of 700 ml for the fermentations on LFM medium, and 750 ml for the fermentations on TP. The LFM experiments were conducted at a stirrer speed of 180 rpm and the initial pH values of the media were 5.55 and 5.58 on LFM1 and LFM2, respectively. The TP experiments were conducted at 210 rpm and the pH was initially adjusted to 5.56, as to standardize the starting conditions between the broths (LFM1 and LFM2) and the TPs (TP1 and TP2), by the addition of NaOH 5 M. The pH was controlled only in this initial phase, and left free during the fermentation. Each test was performed in triplicate, through repeated fermentations. These different stirrer speeds were chosen on the basis of the different viscosity values observed on the two types of substrate and on the basis of a prior study (Gallo et al., 2018). Briefly, for LFM medium (with a viscosity very close to 1.15 [Pa s]) a stirrer speed of 180 rpm was chosen; for TP media (diluted 1:1 with water as previously described, with a viscosity of 1.40 [Pa s], slightly higher than LFM medium) a stirrer speed of 210 rpm was chosen. These stirrer speeds were considered sufficient to guarantee a perfect mixing and therefore homogeneity, as demonstrated by preliminary mixing tests conducted with a food dye (not shown).

2.5 | Analytical methods

Samples were aseptically taken from the bioreactor at specific sampling times (T0, T24, T48, T72, T96, T120, T144, T168, and T192). Those samples were used for pH measurements and for microbiological and chemical analyses. The microbiological analysis was conducted for all the samples by growth on Petri dish; in particular, after serial dilutions, samples were sowed on Petri plate, prepared with YM agar (YM broth, BD 271120 added with 20 g/L of Agar, Sigma-Aldrich); to assess the microbiological growth the plates were incubated for 48 hr at 30°C, in aerobic conditions. Also the optical density was evaluated by a reading at 600 nm. Optical density was evaluated only on LFM1 and LFM2 samples, but not on TP1 and TP2 because of the impossibility of performing optical analysis on TP. To verify the presence of contaminants, the samples were also sowed on Petri plate prepared with McConkey Agar (Oxoid), Gelatin Peptone Agar (Oxoid), and MRS Agar (Oxoid), respectively used for the growth of cocci, for the total bacterial count, and for the growth of lactobacilli. In line pH measurements were performed through a pH meter Mettler Toledo (M300 analytical transmitter equipped with an INPRO3100I pH Sensor). Residual glucose from fermentation process was analyzed by the D-glucose assay procedure (Megazyme). Concentrations of erythritol and citric acid were determined by high performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H, 300 mm × 7.8 mm column (Bio-Rad) which was eluted with H_2O, at 0.6 ml/min flow rate at room temperature. A fixed volume of 20 µl was manually injected and a refractive index detector was used for detection of products.

2.6 | Kinetic parameters

The study continued with the evaluation of kinetic parameters such as the citric acid/erythritol ratio, assessed for each time; the product yield, calculated as following: $Y_p = \Delta P/\Delta S = (P_{out} - P_0)/(S_{out} - S_0)$; where P_{out} was the concentration of the fermentation product at the end of the process, P_0 was the initial concentration of the fermentation product, S_{out} was the concentration of the fermentation substrate at the end of the process, S_0 was the initial concentration of the fermentation substrate; erythritol and citric acid production rate, evaluated as following: $(P_{T_n} - P_{T_{n-1}})$. Finally, the cumulative value for each time was measured, as following: $(P_{T_{n-1}}) + ([P_{T_n} - P_{T_{n-1}}]/P_{T_{192}})$.

3 | RESULTS

3.1 | Microbial growth curve

As shown in Figure 1a the initial microbial load was similar between the different samples and was of about $5.5 \times 10^5 \pm 5 \times 10^4$ CFU/ml. No significant differences were observed between LFM1 and LFM2 in terms of microbial growth. The lag phase lasted 4 hr for both, the exponential growth phase ended at about 24 hr for both and, at that time, a mean charge of about 8.7×10^6 CFU/ml was found (t_{24} bacterial concentration on LFM1 was $1.01 \times 10^7 \pm 1.31 \times 10^6$ CFU/ml, t_{24} bacterial concentration on LFM2 was $7.25 \times 10^6 \pm 1.32 \times 10^6$ CFU/ml); the stationary phase lasted from 24 to 168 hr of fermentation and the death phase began at 168 hr for both. At the end of the process (192 hr) a mean value of about 4×10^6 CFU/ml was found (t_{192} bacterial concentration on LFM1 was $5.45 \times 10^6 \pm 1.05 \times 10^6$ CFU/ml; t_{192} bacterial concentration on LFM2 was $2.9 \times 10^6 \pm 9.6 \times 10^5$ CFU/ml). Conversely, as it is possible to note in Figure 1a, TP1 and TP2 had the same lag phase length (4 hr) respect to LFM1 and LFM2, but differences were notable on their growth curve. On TP1 the exponential growth phase ended at about 24 hr and at that time a charge of about $2.25 \times 10^6 \pm 7.23 \times 10^5$ CFU/ml was found; the stationary phase lasted from 24 to 72 hr of fermentation. The death phase began at 72 hr. At the end of the process (192 hr) $2.68 \times 10^5 \pm 8.06 \times 10^4$ CFU/ml was found. Concerning TP2, the exponential growth phase ended after 48 hr and, at that time, a charge of about $4.8 \times 10^6 \pm 1.15 \times 10^6$ CFU/ml was found; the stationary phase began at 48, but at 192 hr (time at which the fermentation was stopped), the death phase was not yet observable. At 192 hr a microbial charge of $5.3 \times 10^6 \pm 1.01 \times 10^6$ was found. The trend of the optical density in function of fermentation time was also evaluated for LFM1 and LFM2 and is shown in Figure 1b. In this case as well it was possible to define a lag phase, in the first 4 hr of the process, and an exponential growth phase for both. For the stationary

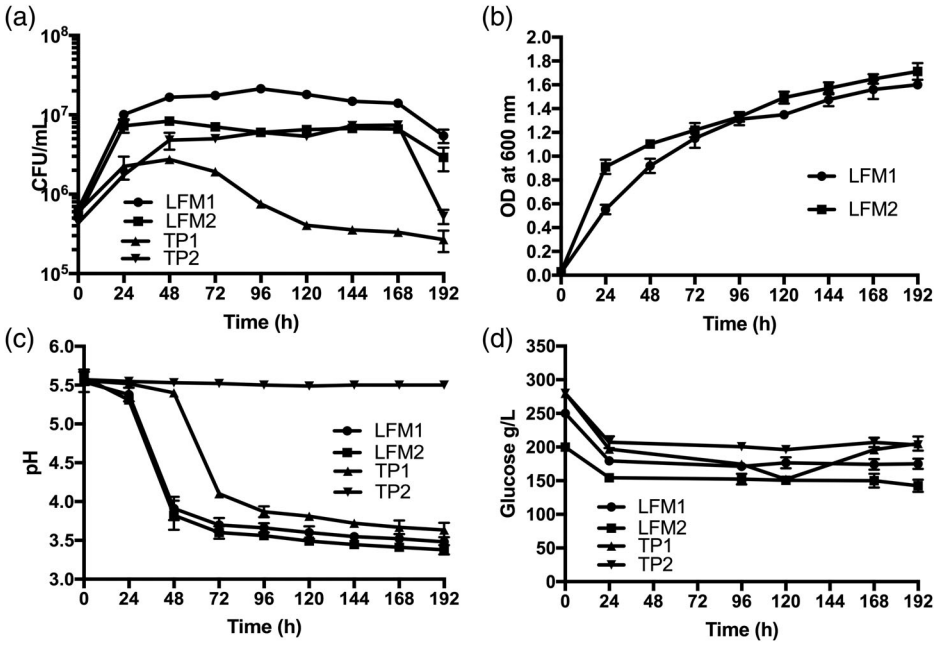

FIGURE 1 Growth curve, pH trend and glucose consumption obtained during *Yarrowia lipolytica* fermentation. (a) Growth curve analysis by culture on petri dish on LFM1, LFM2, TP1, and TP2. (b) Growth curve analysis by optical density on LFM1 and LFM2. (c) pH trend during *Y. lipolytica* fermentation on LFM1, LFM2, TP1, and TP2. (d) Glucose consumption (g/L) overtime on LFM1, LFM2, TP1, and TP2. Each test was performed in triplicate

and the death phases, no correspondences were found between the two methods. No contamination was recognized on McConkey Agar, Gelatin Peptone Agar, and MRS Agar.

3.2 | pH

LFM1, LFM2, TP1, and TP2 samples had the same pH value of about 5.5 at the beginning of the fermentation process, as shown in Figure 1c. The pH value remained constant during the lag phase for all the samples. LFM1 and LFM2 had a pH reduction after 24 hr of fermentation, reaching a mean pH value at t_{48} of about 3.85 (t_{48} pH observed on LFM1 was of 3.9 ± 0.15, while on LFM2 was of 3.8 ± 0.19). That value remained constant until the end of the process, as shown in Figure 1c. Conversely, TP1 had a constant pH value until 48 hr. After that time a strong pH reduction up to a value of 3.63 ± 0.10 was observed. The TP2's pH trend remained constant from the beginning of the fermentation process until the end.

3.3 | Glucose analysis

The glucose reduction overtime is shown in Figure 1d. In particular, LFM1 started at T_0 with a glucose concentration of 250 ± 2 g/L, defined in the culture medium, and a progressive reduction overtime, up to a concentration of 175 ± 8 g/L, was observed at the end of the process (192 hr). The overall percentage decrease was 30%. The LFM2 initial glucose concentration was of about 200 ± 2 g/L, as defined in the culture medium, and a progressive reduction of the glucose concentration overtime, up to a concentration of 142.4 ± 9 g/L, was observed at the end of the process (192 hr). The overall percentage decrease was 29%. TP1 and TP2 had a glucose concentration at T_0 of about 280 ± 3 g/L, as defined in the culture medium. The glucose concentration at the end of the process was very similar between TP1 and TP2 (205.2 ± 10.4 g/L and 203 ± 4.6 g/L, respectively). However, an overall reduction of about 30% at the end of the process (192 hr) was found in all the samples tested (LFM1, LFM2, TP1, and TP2).

3.4 | Erythritol and citric acid analysis

The trend of erythritol production, measured by HPLC, during LFM1, LFM2, TP1, and TP2 fermentation in function of time is shown in Figure 2a. The trends of erythritol production related to all the samples increased overtime, up to a maximum value reached in the final phase of the process (after 192 hr), which for LFM1 was 1.18 ± 0.04 g/L, for LFM2 was 0.53 ± 0.05 g/L, for TP1 was 1.6 ± 0.03 g/L, and for TP2 was 0.72 ± 0.04 g/L. Citric acid production is shown in Figure 2b. In this case as well an increasing trend overtime was found for all the samples. The maximum value was indeed reached at 192 hr and corresponded to 6.075 ± 0.06 g/L for LFM1, 4.325 ± 0.09 g/L for LFM2, 11.585 ± 0.61 g/L for TP1, and 2.725 ± 0.07 g/L for TP2. To note that, since the TP already contained citric acid, at T0 a concentration of citric acid equal to 1.14 ± 0.05 g/L was found on TP1 and 1.14 ± 0.07 g/L was found on TP2. To facilitate comparisons, in Figure 2c the concentrations of erythritol and citric acid obtained on broths (LFM1 and LFM2) were combined, while in Figure 2d those obtained on TPs (TP1 and TP2). An exemplary chromatogram is shown in Figure S1.

3.5 | Kinetic parameters

Citric acid/erythritol concentration ratio for each time is shown in Figure 2e. As it is possible to note, for each sample the reaction was pushed towards the production of citric acid, rather than of erythritol. These ratios remained constant for LFM2, TP1, and TP2, while for

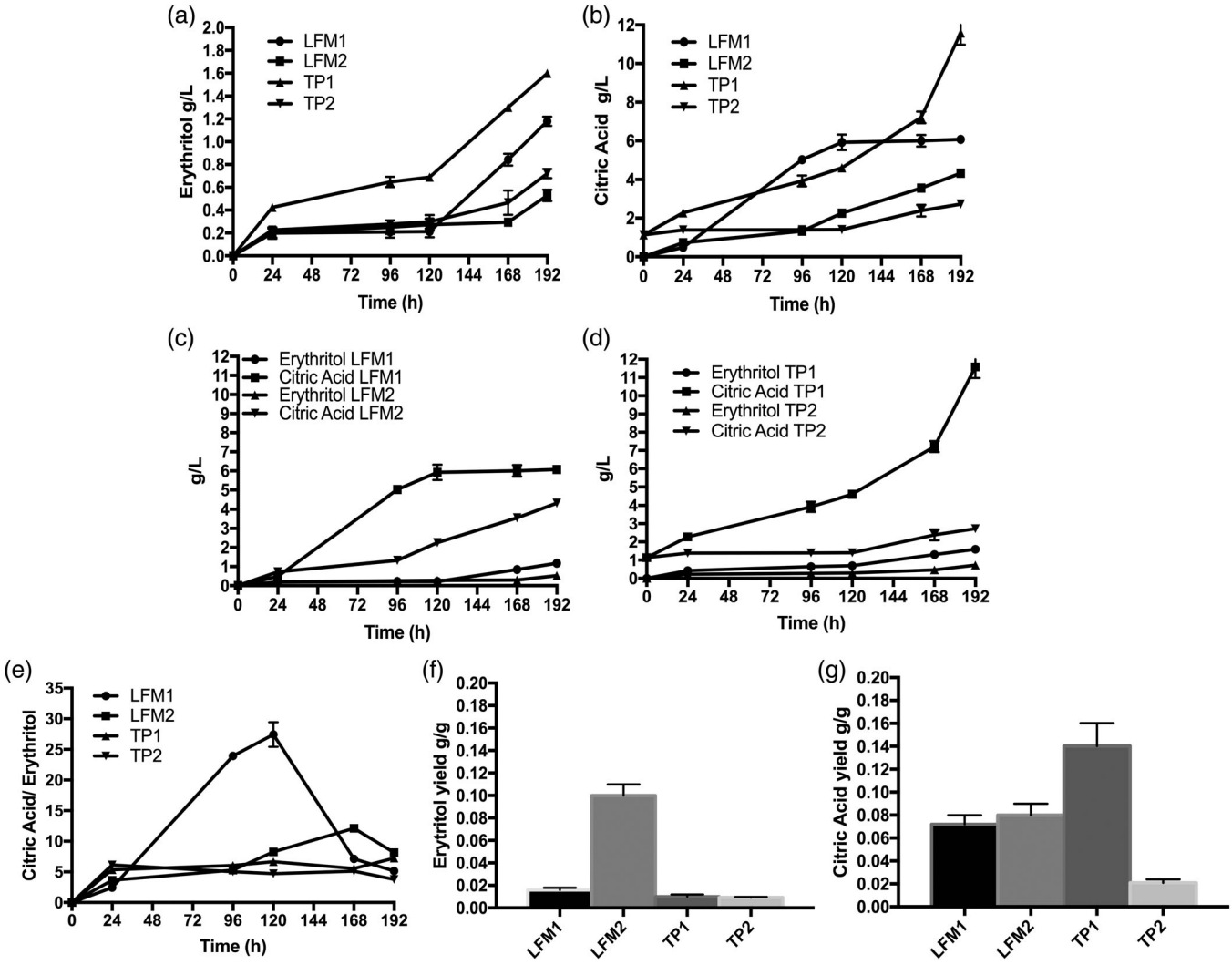

FIGURE 2 Products concentrations and yields obtained during *Yarrowia lipolytica* fermentation. (a) Erythritol concentration (g/L) obtained during LFM1, LFM2, TP1, and TP2 fermentations. (b) Citric acid concentration (g/L) obtained during LFM1, LFM2, TP1, and TP2 fermentations. (c) Erythritol and citric acid concentrations (g/L) obtained on LFM1 and LFM2 broths. (d) Erythritol and citric acid concentrations (g/L) obtained on TP1 and TP2. (e) Citric acid/erythritol ratios obtained during LFM1, LFM2, TP1, and TP2 fermentation. (f) Erythritol yield (g/g) obtained during LFM1, LFM2, TP1, and TP2 fermentations. (g) Citric acid yield (g/g) obtained during LFM1, LFM2, TP1, and TP2 fermentations. Each test was performed in triplicate

TABLE 1 Analytical parameters found for LFM1, LFM2, TP1, TP2, and related erythritol and citric acid yields

	G_0 (g/L)	G_{out} (g/L)	E_0 (g/L)	E_{out} (g/L)	A_0 (g/L)	A_{out} (g/L)	Y_E (g/g)	Y_{AC} (g/g)
LFM1	250 ± 2	175 ± 7.6	–	1.18 ± 0.04	–	6.075 ± 0.06	0.016 (1.6%)	0.072 (7.2%)
LFM2	200 ± 2	142.38 ± 9.1	–	0.53 ± 0.05	–	4.33 ± 0.09	0.098 (0.98%)	0.08 (8%)
TP1	280 ± 4	205.2 ± 10.4	–	0.16 ± 0.03	1.14 ± 0.05	11.59 ± 0. 61	0.01 (0.2%)	0.14 (14%)
TP2	280 ± 3	203 ± 4.6	–	0.72 ± 0.04	1.14 ± 0.07	2.73 ± 0.07	0.009 (0.9%)	0.021 (2.1%)

LFM1 a rapid change in the production of citric acid could be observed, from 24 to 120 hr. Products yields were calculated as described in Section 2. Initial glucose concentration (G_0), final glucose concentration (G_{out}), initial erythritol concentration (E_0), final erythritol concentration (E_{out}), the initial citric acid concentration (A_0), the final citric acid concentration (A_{out}), and the related erythritol and citric acid

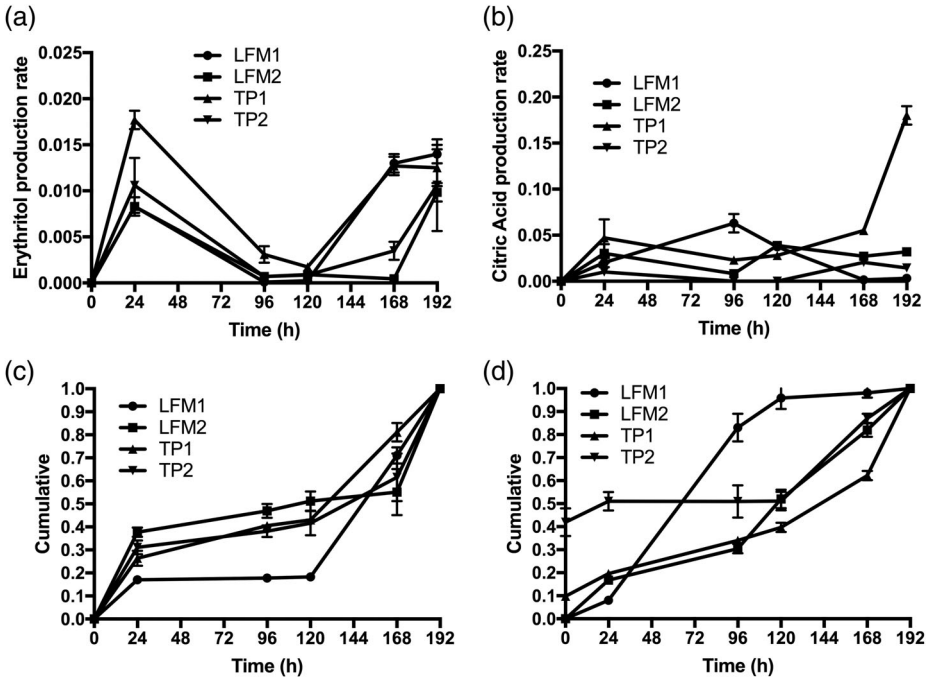

FIGURE 3 Erythritol and citric acid production rate. (a) Erythritol production rate during LFM1, LFM2, TP1, and TP2 fermentations. (b) Citric acid production rate during LFM1, LFM2, TP1, and TP2 fermentations. (c) Erythrirol cumulative value overtime during LFM1, LFM2, TP1, and TP2 fermentations. (d) Citric acid cumulative values overtime during LFM1, LFM2, TP1, and TP2 fermentations. Each test was performed in triplicate

yields (Y_E and Y_{AC}) obtained are summarized in Table 1, for all the samples tested (LFM1, LFM2, TP1, and TP2). The product yields are also shown in Figure 2f (erythritol yield) and Figure 2g (citric acid yield). Erythritol and citric acid production rate and their cumulative values are shown in Figure 3. The erythritol production rate for each sample and relative time is shown in Figure 3a. As it is possible to note, for each sample (LFM1, LFM2, TP1, and TP2) an increase in the erythritol production rate was evident at 24 hr. This increase was very similar between LFM1, LFM2, and TP2, while at the same time for TP1 a higher production rate respect to the other samples was registered. Similar erythritol production rates were measured at 120 hr. After that time LFM1 and TP1 showed an increase, while LFM2 and TP2 showed the same increase but after 168 hr. The citric acid production rate for each sample and relative time is shown in Figure 3b. As it is possible to note, for each sample (LFM1, LFM2, TP1, and TP2) no significant difference was notable until 168 hr. After that time only for TP1 a higher production rate was evident. As for the cumulative values, to be considered as the increase of product at each time point compared to the final product, they are shown in Figure 3c (for the erythritol) and in Figure 3d (for the citric acid).

4 | DISCUSSION

Y. lipolytica is a yeast with great biotechnological potential in food industry, found naturally in foods with high proportions of fat and/or protein. Generally it is recognized as safe to use for food application (Groenewald et al., 2013); for this reason and because it seems to be a promising microorganism for the production of citric acid and erythritol, it was chosen in the present study, aimed at understanding the influencing factors on yeast growth and metabolism, paying particular attention to the coproduction of erythritol and citric acid. From literature it is possible to affirm that under stress condition the yeast can react by producing specific metabolites. For example, as studied by Yang et al. (2014, 2015) and Yang, Zhan, Zhu, Gao, and Lin (2016), the hyperosmotic stress, caused by the excess of the carbon source or by salts addition, could let the microorganism to produce metabolites such erythritol. Another metabolic stress able to induce specific metabolites production is linked to the nitrogen content: the absence of nitrogen in the fermentation medium causes an early death phase for the yeast (Behrens, Thiersch, Weissbrodt, & Stottmeister, 1987) and, at the same time, a higher citric acid production. Thus, nitrogen limitation gradually turns yeast metabolism from cellular growth to product formation (Anastassiadis, Aivasidis, & Wandrey, 2002). Furthermore, few works showed that some polyols accumulate during citric acid fermentation (Gutierrez-Rojas, Còrdova, Auria, Revah, & Favela-Torres, 1995; Rohr, Kubicek, Zehentgruber, & Orthofer, 1987; Rymowicz, Fatykhova, Kamzolova, Rywińskalgor, & Morgunov, 2010). Erythritol, in particular, seemed to accumulate during the later stages of fermentation (Rohr et al., 1987). From literature is also possible to affirm that another important factor influencing the erythritol and citric acid production is pH. In fact, *Y. lipolytica*, in response to a stress such as pH lowering, is able to respond by increasing the synthesis of erythritol (Rymowicz, Rywiska, & Marcinkiewicz, 2009; Tomaszewska, Rywinska, & Rymowicz, 2014). Rymowicz, in particular showed how maintaining the pH at low values, the erythritol production increased while that of citric acid decreased. The combined effects of osmotic pressure and pH was also investigated (Liu et al., 2018): increasing the osmotic stress with a low pH, caused an increased production of erythritol but low levels of citric acid; on the contrary, with reduced osmotic stress and high pH the situation changed. As hyperosmotic agents we used an excess of glucose, which by itself, was not able to sufficiently stress the yeast in order to induce it to produce

TABLE 2 Summary from the literature of processes used and relative results in term of erythritol and citric acid production

	Process conditions							Results				
Strain	pH	T (°C)	Substrate	Agitation (rpm)	Time (hr)	Flask	Fermentor	Erythritol (g/L)	Ye (g/g)	Citric acid (g/L)	Yca (g/g)	Reference
C. magnoliae M2(mutant)	Na	28	20 g/L glucose	200	48	500 mlL flasks containing 200 mL	–	25	0.25	–	–	Yang et al. (1999)
	pH 0:7	28	200 g/L glucose	700	192		Batch in 2.5 L jar fermentor; working volume of 1 L	85	0.43	–	–	
C. magnoliae (mutant)	pH 0:7 pHf: 3.2	28	400 g/L glucose	Controlled to have oxygen level above 20%	60	–	Fed batch in 3.3 L jar fermentor	187	0.41	65	–	Ryu, Park, Park, Kim, and Seo (2000)
Yarrowia lipolytica ATCC 20346	pH: 5	30	250 g/L glucose	900	70	–	Batch in 15 L stirred tank fermentor	–	–	–	0.384	Antonucci et al. (2001)
Y. lipolytica LGAM S (7)1	pH 0:6.5 pHf: 4.3	28	28 g/L glucose	185 rev min^{-1}	140	250 ml flasks containing 50 ml	–	–	–	11	0.39	Papanikolaou et al. (2002)
	pH 0:6.5 pHf: 4.5		31 g/L glycerol							12	0.38	
Y. lipolytica Wratislavia K1(mutant)	pH: 5.5	30	250 g/L glycerol	600	168	–	Fed batch in a 3.5 L reactor; working volume of 1.3 L	81	0.32	110	0.44	Rymowicz et al. (2008)
Y. lipolytica Wratislavia K1(mutant)	pH: 3	30	300 g/L glycerol	800	168	–	Fed batch in a 5 L jar fermentor; working volume of 2 L	170	0.56	0	–	Rymowicz et al. (2009)
Y. lipolytica A-101 (wt)								137	0.46	22	–	
Y. lipolytica 1.22 (wt)								93.5	0.31	54	–	
Y. lipolytica Wratislavia 1.31 (mutant)	pH: 5.5	30	200 g/L glycerol	600	506	–	Cell recycle system in a 5 L jar fermentor; working volume of 1.4 L	5.2	–	96	0.51	Rywińska and Rymowicz (2011)
Y. lipolytica Wratislavia AWG7 (mutant)					550			4.1	–	112	0.54	

(Continues)

TABLE 2 (Continued)

Strain	Process conditions							Results				Reference
	pH	T (°C)	Substrate	Agitation (rpm)	Time (hr)	Flask	Fermentor	Erythritol (g/L)	Ye (g/g)	Citric acid (g/L)	Yca (g/g)	
Y. lipolytica A UV'1 (mutant)	Na	29.5	100 g/L glycerol	140	240	0.3 L flasks containing 0.03 L	–	27.5	0.38	–	–	Tomaszewska et al. (2012)
	pH: 3	29.5	150 g/L glycerol	800	Until substrate consumption	–	Batch in 5 L stirred-tank reactor, working volume of 2.0 L	59.3	0.35	–	–	
	pH: 3	29.5	250 g/L glycerol	800	Until substrate consumption	–	Batch in 5 L stirred-tank reactor, working volume of 2.0 L	91.6	0.37	–	–	
Y. lipolytica A-15 (wt)	Na	29.5	100 g/L glycerol	140	240	0.3 L flasks containing 0.03 L	–	35.53	0.42 substrat	–	–	
	pH: 3	29.5	150 g/L glycerol	800	Until substrate consumption	–	Batch in 5 L stirred-tank reactor; working volume of 2.0 L	28	0.19	–	–	
	pH: 3	29.5	250 g/L glycerol	800	Until substrate consumption	–	Batch in 5 L stirred-tank reactor; working volume of 2.0 L	66.3	0.27	–	–	
Y. lipolytica Wratislavia K1 (mutant)	Na	29.5	100 g/L glycerol	140	240	0.3 L flasks containing 0.03 L	–	23.23	0.43	–	–	
	pH: 3	29.5	150 g/L glycerol	800	Until substrate consumption	–	Batch in 5 L stirred-tank reactor; working volume of 2.0 L	42	0.22	–	–	
	pH: 3	29.5	250 g/L glycerol	800	Until substrate consumption	–	Batch in 5 L stirred-tank reactor; working volume of 2.0 L	Na	Na	–	–	
Y. lipolytica Wratislavia K1 (mutant)	pH: 3	30	250 g/L glycerol	800	1,103	–	Repeated batch culture in 5 L jar fermentor; working volume of 2 L	220	0.43	1.1	–	Mirończuk et al. (2014)

(Continues)

TABLE 2 (Continued)

Strain	Process conditions							Results				Reference
	pH	T (°C)	Substrate	Agitation (rpm)	Time (hr)	Flask	Fermentor	Erythritol (g/L)	Ye (g/g)	Citric acid (g/L)	Yca (g/g)	
Y. lipolytica CICC 1675 (wt)	pH: 3	30	200 g/L glycerol	800	132	–	Fed batch in 7 L stirred-tank reactor; working volume of 3 L	194.3	0.49	–	–	Yang et al. (2014)
Y. lipolytica Wratislavia K1 (mutant)	pH: 3	30	200 g/L glycerol	13.3 Hz	850	–	Fed batch in 5 L stirred tank reactor; working volume of 1.5 L	103.4	0.52	0.7	–	Rakicka et al. (2016)
Y. lipolytica DSM 3286	pH: 2.5	30	200 g/L glycerol	180	72	–	Batch in 1.2 L reactor; working volume of 0.5 L	Na	0.41[a]	Na	0[b]	Egermeier, Russmayer, Sauer, and Marx (2017)
	pH: 3							Na	0.43[a]	Na	0.02[b]	
	pH: 3.5							Na	0.36[a]	Na	0.04[b]	
	pH: 4.5							Na	0.20[a]	Na	0.34[b]	
	pH: 5.5							Na	0.13[a]	Na	0.44[b]	
	pH: 6.5							Na	0.09[a]	Na	0.42[b]	
	pH: 7.5							Na	0.08[a]	Na	0.4[b]	

[a]intended as polyols yield.
[b]intended as yield citric acid/isocitric acid.

metabolites, and this was evident considering the yeast growth, the citric acid production, the pH, and the erythritol production obtained on TP2. So adding as another element of stress, the absence of nitrogen source, the yeast went early to the death phase, as demonstrated on TP1. Moreover, a higher citric acid production was obtained, with a consequently pH reduction, by which, as found in literature, a higher erythritol production was obtained. Looking more carefully at the diagrams, it is also possible to note that the stationary phase on TP1 lasted from 24 to 72 hr (while on TP2 it lasted up to 168 hr), with a significantly lower charge. From 72 hr it is also possible to distinguish two different slopes in the decrease of the microbial load: the first from 72 to 120 hr and the second from 120 to 192 hr. The same trend is present on the citric acid' and on the erythritol production graphs. Novelties of the present work can be summarized in several points. Firstly, a wild type strain was used. *Y. lipolytica* was used as strain in several works, but, usually, it was mutagenized (Ghezelbash, Nahvi, & Emamzadeh, 2014; Mirończuk, Rakicka, & Rymowicz, 2014; Rakicka, Rukowicz, Rywinska, Lazar, & Rymowicz, 2016; Rymowicz, Rywiska, & Gadkowski, 2008; Rywińska, Marcinkiewicz, Cibis, & Rymowicz, 2015; Rywińska & Rymowicz, 2011). Rymowicz et al. (2009), Tomaszewska, Rywinska, and Gładkowski (2012), Yang et al. (2014), and Rakicka, Rywinska, Lazar, and Rymowicz (2017) used *Y. lipolytica* genetically unmodified to produce erythritol, but in all cases glycerol was used instead of glucose as a carbon source. Furthermore in none of these works citric acid was an expected product, thus it was not analyzed or was considered as a by-product. In the same way a wild type *Y. lipolytica* was used as fermenting strain to produce citric acid, but also in this case erythritol was considered as a by-product (Rzechonek, Dobrowolski, Rymowicz, & Mirończuk, 2019). Secondly, to avoid the extraction phase necessary if the production should be performed on a simple broth, the ability of the strain to produce erythritol and citric acid was tested directly into the food matrix. To the best of our knowledge in no other study this was tried. On the contrary, several works on the erythritol or citric acid production on specific broth were found in literature (Yang, Park, Han, Ryu, & Seo, 1999; Antonucci, Bravi, Bubbico, Di Michele, & Verdone, 2001; Papanikolaou, Muniglia, Chevalot, Aggelis, & Marc, 2002; Tomaszewska et al., 2012; Yang, 2014), and results of these works are summarized in Table 2. Furthermore, a simultaneous production of erythritol and citric acid was performed in the present work. Other publications, also summarized in Table 2, had the same purpose. For example, Ryu et al. (2000) obtained through a fed-batch fermentation by a mutant *Candida magnoliae*, the production of both erythritol (187 g/L) and citric acid (65 g/L), starting with 400 g/L of glucose. In the same way, Rymowicz et al. (2008), using a mutagenized *Y. lipolytica*, obtained 81 g/L of erythritol and 110 g/L of citric acid through a fed-batch fermentation; Rymowicz et al. (2009)) used a mutagenized *Y. lipolytica*, obtaining only the erythritol production; he used also two wild type strains (*Y. lipolytica A-101* and *Y. lipolytica 1.22*) obtaining a production of erythritol lower than that obtained with the mutant, but citric acid was also produced. Rywińska and Rymowicz (2011) used a mutagenized *Y. lipolytica* and, through a cell recycle system, obtained the co-production of erythritol and citric acid, leaving the strain to ferment for more than 500 hr. Rakicka et al. (2016) used a mutagenized *Y. lipolytica*, obtaining a great erythritol production. However citric acid production was low. Egermeier et al. (2017) tested the ability of several wild type strains of *Y. lipolytica* in producing citric acid and polyols on selected broth. In particular crude glycerol was used as carbon source and different pH values were screened (for simplicity only one strain of the mentioned study was reported in Table 2).

We are aware that the main limit of our work is the low yield compared to the high concentration of glucose used, considering the desire to directly produce, through fermentation, a semifinished product containing erythritol and citric acid. Our future perspective will be the reduction of the carbon source, trying to use as hyperosmotic agents other compounds such as salts (Yang et al., 2015). Another influential factor to take into account will also the pH control, in order to direct the metabolism towards the production of citric acid or erythritol. Furthermore, aspect to be considered for future trials will be a broader analysis, to detect the possible presence of metabolites other than erythritol and citric acid. *Y. lipolytica* in fact has the ability to produce a wide variety of molecules. Examples of these are other polyols, as mannitol or arabitol (Mirończuk et al., 2014; Yang et al., 2014); other organic acids, as isocitric acid (Kamzolova et al., 2013), α-ketoglutaric acid (Yovkova, Otto, Aurich, Mauersberger, & Barth, 2014), pyruvic acid (Zhou, Yin, Madzak, Du, & Chen, 2012), and succinic acid (Kamzolova et al., 2014); proteins as lipase, protease, and esterase (Liu et al., 2015); aromatic compounds as γ-decalactone (Moradi, Asadollahi, & Nahvi, 2013) and so on. Finally, considering the wide variety of molecules that *Y. lipolytica* can produce (with possible influence on the organoleptic properties of the product) and taking in account that some strains can be isolated from patients (even if opportunistic infections from *Y. lipolytica* occur very rarely), performing safety, shelf life, and sensory tests on products of *Y. lipolytica* will be very important.

5 | CONCLUSIONS

A batch fermentation by a non-mutagenized *Y. lipolytica* on simple broth and on a more complex substrate (diluted TP), was performed. In all the fermentations, erythritol, a natural sweetener, and citric acid, a natural preservative, were obtained. With the present study we demonstrated the possibility to coproduce erythritol and citric acid; moreover we observed that the citric acid production and thus the erythritol production were related to the yeast death, due to the metabolic stress induced by both hyperosmosys and nitrogen deficiency. Therefore, influencing factors for the production of erythritol and citric acid seemed to be the glucose concentration, acting as a factor promoting the hyperosmotic stress, the nitrogen amount and the pH. This study can be considered a first step for the development of new processes aimed at obtaining foods with reduced content in sugars, but maintaining the same organoleptic properties and the same shelf life. Future perspectives should be the improvement of

the process, for example by reducing the glucose concentration, by the addition of other hyperosmotic factors, or by controlling the pH during the fermentation. In particular, with a better understanding of *Y. lipolytica*'s metabolic pathways and with specific modulation of influencing factors (i.e., glucose, nitrogen, time, pH, etc.) we could be able to direct the metabolism to the citric acid's and/or erythritol's production. Moreover, the effective sweetening capacity of erythritol and the preservative power of citric acid produced by fermentation should be tested by specific analysis, in conjunction with the safety assessment of the semifinished product obtained.

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REFERENCES

Anastassiadis, S., Aivasidis, A., & Wandrey, C. (2002). Citric acid production by *Candida* strains under intracellular nitrogen limitation. *Applied Microbiology and Biotechnology*, *60*, 81–87.

Antonucci, S., Bravi, M., Bubbico, R., Di Michele, A., & Verdone, N. (2001). Selectivity in citric acid production by *Yarrowia lipolytica*. *Enzyme and Microbial Technology*, *28*(2), 189–195.

Behrens, U., Thiersch, A., Weissbrodt, E., & Stottmeister, U. (1987). Particularities in the kinetics of growth and citric acid accumulation by *Saccharomycopsis lipolytica*. *Acta Biotechnologica*, *7*, 179–183.

Cavallo, E., Charreau, H., Cerrutti, P., & Foresti, M. L. (2017). *Yarrowia lipolytica*: A model yeast for citric acid production. *FEMS Yeast Research*, *17*(8).

Egermeier, M., Russmayer, H., Sauer, M., & Marx, H. (2017). Metabolic flexibility of *Yarrowia lipolytica* growing on glycerol. *Frontiers in Microbiology*, *8*, 49.

Flint, N., Hamburg, N., Holbrook, M., Dorsey, P., LeLeiko, R., Berger, A., … Vita, J. (2013). Effects of erythritol on endothelial function in patients with type 2 diabetes mellitus: A pilot study. *Acta Diabetologica*, *51*, 513–516.

Foreign Agricultural Service/USDA, (2017). *Sugar: World Markets and Trade*. Washington, DC: Foreign Agricultural Service/USDA.

Gallo, M., Nigro, F., Passannanti, F., Salameh, D., Budelli, A., Marzocchella, A., & Nigro, R. (2018). Rice Fermentation by *Lactobacillus paracasei CBA L74*. *International Journal of Rice Research*.

Ghezelbash, G. R., Nahvi, I., & Emamzadeh, R. (2014). Improvement of erythrose reductase activity, deletion of by-products and statistical media optimization for enhanced erythritol production from *Yarrowia lipolytica* mutant 49. *Current Microbiology*, *69*(2), 149–157.

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., van Dijck, P. W. M., & Wyss, M. (2013). *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. *Critical Review in Microbiology*, *40*(3), 187–206.

Gutierrez-Rojas, M., Còrdova, J., Auria, R., Revah, S., & Favela-Torres, E. (1995). Citric acid and polyols production by aspergillus Niger at high glucose concentration in solid state fermentation on inert support. *Biotechnology Letters*, *17*(2), 219–224.

Kamzolova, S. V., Dedyukhina, E. G., Samoilenko, V. A., Lunina, J. N., Puntus, I. F., Allayarov, R. L., … Morgunov, I. G. (2013). Isocitric acid production from rapeseed oil by *Yarrowia lipolytica* yeast. *Applied Microbiology and Biotechnology.*, *97*, 9133–9144.

Kamzolova, S. V., Vinokurova, N. G., Dedyukhina, E. G., Samoilenko, V. A., Lunina, J. N., Mironov, A. A., … Morgunov, I. G. (2014). The peculiarities of succinic acid production from rape-seed oil by *Yarrowia lipolytica* yeast. *Applied Microbiology and Biotechnology.*, *98*, 4149–4157.

Liu, H. H., Ji, X. J., & Huang, H. (2015). Biotechnological applications of *Yarrowia lipolytica*: Past, present and future. *Biotechnology Advances*, *33*, 1522–1546.

Liu, X., Lv, J., Xu, J., Xia, J., He, A., Zhang, T., … Xu, J. (2018). Effects of osmotic pressure and pH on citric acid and erythritol production from waste cooking oil by *Yarrowia lipolytica*. *Engineering in Life Sciences*, *18*(6), 344–352.

Mirończuk, A. M., Rakicka, J. F. M., & Rymowicz, W. (2014). Enhanced production of erythritol by *Yarrowia lipolytica* on glycerol in repeated batch cultures. *Journal of Industrial Microbiology & Biotechnology*, *41*, 57–64.

Moon, H. J., Jeya, M., Kim, I. W., & Lee, J. K. (2010). Biotechnological production of erythritol and its applications. *Applied Microbiology and Biotechnology*, *86*(4), 1017–1025.

Moradi, H., Asadollahi, M. A., & Nahvi, I. (2013). Improved γ-decalactone production from castor oil by fed-batch cultivation of *Yarrowia lipolytica*. *Biocatalysis and Agricultural Biotechnology.*, *2*, 64–68.

Otey, F. H., Sloan, J. W., Wilham, C. A., & Mehltretter, C. L. (1961). Erythritol and ethylene glycol from dialdehyde starch. *Industrial and Engineering Chemistry*, *53*(4), 267–268.

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., & Marc, I. (2002). *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. *Journal of Applied Microbiology*, *92*, 737–744.

Rakicka, M., Rukowicz, B., Rywinska, A., Lazar, Z., & Rymowicz, W. (2016). Technology of efficient continuous erythritol production from glycerol. *Journal of Cleaner Production*, *139*, 905e913.

Rakicka, M., Rywinska, A., Lazar, Z., & Rymowicz, W. (2017). Two-stage continuous culture e technology boosting erythritol production. *Journal of Cleaner Production*, *168*, 420e427.

Regnat, K., Mach, R. L., & Mach-Aigner, A. R. (2018). Erythritol as sweetener—Wherefrom and whereto? *Applied Microbiology and Biotechnology*, *102*(2), 587–595.

Rohr, M., Kubicek, C. P., Zehentgruber, O., & Orthofer, R. (1987). Accumulation and partial re-consumption of polyols during citric acid fermentation by *Asperyillus Niger*. *Applied Microbiology and Biotechnology.*, *27*(3), 235–239.

Runnel, R., Makinen, K.K., Honkala, S., Olak, J., Mäkinen, P.L., Nõmmela, R., Vahlberg T., Honkala and Saag, M., (2013). Effect of three-year consumption of erythritol, xylitol and sorbitol candies on various plaque and salivary caries-related variables. *Journal of Dentistry*. *41*: 1236–1244.

Rymowicz, W., Fatykhova, A. R., Kamzolova, S. V., Rywińskalgor, A., & Morgunov, I. G. (2010). Citric acid production from glycerol-containing waste of biodiesel industry by *Yarrowia lipolytica* in batch, repeated batch, and cell recycle regimes. *Applied Microbiology and Biotechnology*, *87*(3), 971–979.

Rymowicz, W., Rywiska, A., & Gadkowski, W. (2008). Simultaneous production of citric acid and erythritol from crude glycerol by *Yarrowia lipolytica Wratislavia K1*. *Chemical Papers*, *62*(3), 239–246.

Rymowicz, W., Rywiska, A., & Marcinkiewicz, M. (2009). High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*. *Biotechnology Letters*, *31*(3), 377–380.

Ryu, Y. W., Park, C. Y., Park, J. B., Kim, S. Y., & Seo, J. H. (2000). Optimization of erythritol production by *Candida magnoliae* in fed-batch culture. *Journal of Industrial Microbiology and Biotechnology*, *25*(2), 100–103.

Rywińska, A., Marcinkiewicz, M., Cibis, E., & Rymowicz, W. (2015). Optimization of medium composition for erythritol production from glycerol by *Yarrowia lipolytica* using re-sponse surface methodology. *Preparative Biochemistry and Biotechnology*, *45*, 515–529.

Rywińska, A., & Rymowicz, W. (2011). Continuous production of citric acid from raw glycerol by in cell recycle cultivation. *Chemical Papers*, *65*(2), 119–123.

Rzechonek, D. A., Dobrowolski, A., Rymowicz, W., & Mirończuk, A. M. (2019). Aseptic production of citric and isocitric acid from crude glycerol by genetically modified *Yarrowia lipolytica*. *Bioresource Technology*, *271*, 340–344.

Savergave, L. S. (2011). *Microbial production of erythritol and mannitol: Strain improvement and process optimization*. (Doctoral Dissertation. Pune, India: CSIR-National Chemical Laboratory.

Soccol, C. R., Vandenberghe, L. P. S., & Rodrigues, C. (2006). New perspectives for citric acid production and application. *Food Technology and Biotechnology*, *44*(2), 141–149.

Stanhope, K. L. (2016). Sugar consumption, metabolic disease and obesity: The state of the controversy. *Critical Reviews in Clinical Laboratory Sciences*, *53*(1), 52–67.

Tomaszewska, L., Rywinska, A., & Gładkowski, W. (2012). Production of erythritol and mannitol by *Yarrowia lipolytica* yeast in media containing glycerol. *Journal of Industrial Microbiology & Biotechnology*, *39*, 1333–1343.

Tomaszewska, L., Rywinska, A., & Rymowicz, W. (2014). High selectivity of erythritol production from glycerol by *Yarrowia lipolytica*. *Biomass and Bioenergy*, *64*, 309–320.

Wölnerhanssen, B. K., Cajacob, L., Keller, N., Doody, A., Rehfeld, J. F., Drewe, J., … Meyer-Gerspach, A. C. (2016). Gut hormone secretion, gastric emptying, and glycemic responses to erythritol and xylitol in lean and obese subjects. *American Journal of Physiology-Endocrinology and Metabolism*, *310*, E1053–E1061.

World Health Organization. (2015) *Guideline: sugars intake for adults and children*. Geneva: World Health Organization.

Yang, L. B., Dai, X. M., Zheng, Z. Y., Zhu, L., Zhan, X. B., & Lin, C. C. (2015). Proteomic analysis of erythritol-producing *Yarrowia lipolytica* from glycerol in response to osmotic pressure. *Journal of Microbiology and Biotechnology*, *25*, 1056–1069.

Yang, L. B., Zhan, X. B., Zheng, Z. Y., Wu, J. R., Gao, M. J., & Lin, C. C. (2014). A novel osmotic pressure control fed-batch fermentation strategy for improvement of erythritol production by *Yarrowia lipolytica* from glycerol. *Bioresource Technology*, *151*, 120–127.

Yang, L. B., Zhan, X. B., Zhu, L., Gao, M. J., & Lin, C. C. (2016). Optimization of a low-cost hyperosmotic medium and establishing the fermentation kinetics of erythritol production by *Yarrowia lipolytica* from crude glycerol. *Preparative Biochemistry and Biotechnology*, *46*(4), 376–383.

Yang, S. W., Park, J. B., Han, N. S., Ryu, Y. W., & Seo, J. H. (1999). Production of erythritol from glucose by an osmophilic mutant of *Candida magnoliae*. *Biotechnology Letters*, *21*(10), 887–890.

Yovkova, V., Otto, C., Aurich, A., Mauersberger, S., & Barth, G. (2014). Engineering the α-ketoglutarate overproduction from raw glycerol by overexpression of the genes encoding NADP+-dependent isocitrate dehydrogenase and pyruvate carboxylase in *Yarrowia lipolytica*. *Applied Microbiology and Biotechnology*, *98*, 2003–2013.

Zhou, J., Yin, X., Madzak, C., Du, G., & Chen, J. (2012). Enhanced α-ketoglutarate production in *Yarrowia lipolytica* WSH-Z06 by alteration of the acetyl-CoA metabolism. *Journal of Biotechnology.*, *161*, 257–264.

Zinjarde, S. S. (2014). Food-related applications of *Yarrowia lipolytica*. *Food Chemistry*, *152*(2014), 1–10.

Zink, D. L. (1997). The impact of consumer demands and trends on food processing. *Emerging Infectious Diseases Journal*, *3*(4), 467–469.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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