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Department of Chemical, Materials and Industrial Production Engineering

PhD Thesis in

Industrial Product and Process Engineering

Development of processes for the production of postbiotic functional foods

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Summary

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Abstract

Several techniques were developed in order to enhance the content of bioactive compounds or promote their production in food products. Lactic acid fermentation is one of the most important food processing technologies used to produce functional probiotic foods. A novel category of fermented functional foods is gaining increasing interest and is represented by postbiotic products, consisting of inactivated microorganisms and molecules (enzymes, peptides, organic acids) produced in the final or intermediate stage of the metabolic process of these probiotic bacteria. Although postbiotics do not contain live microorganisms, they show a beneficial health effect through similar mechanisms that are characteristic of probiotics, minimizing the risks associated with their intake. For this reason, they could be considered a safer alternative to clinical application for immune-deficient patients or infants. This research, in collaboration with Kraft Heinz Company, consisted of the development of processes for the production of wet and dried functional foods with a postbiotic effect, intended for categories of people with high pathological vulnerability, such as children, elderly people and pregnant women. The purpose of this project was to investigate the effect of fermentation process, carried out using Lactobacillus paracasei CBA L74, patented by Kraft Heinz, heat treatments and drying processes on the postbiotic properties of the semi-finished products obtained, choosing as raw materials milk, rice flour and leguminous (beans) suspensions. The processes were examined and optimized at laboratory scale and then implemented at pilot scale with a successful and efficient scaling up, except for beans that had been an unexplored food matrix to be fermented until then and required a further optimization of protocols at laboratory scale before performing the trials on the pilot plant.

At lab scale, rice and milk fermentation were carried out with and without pH control and in both cases an improvement in the terms of kinetic performance of the process was observed when pH control was implemented. The fermentation process was characterized in terms of bacterial growth (maximum values of $1.9 \times 10^8 \pm 1.2 \times 10^8$ CFU/mL after 20 h and $5.3 \times 10^8 \pm 4.7 \times 10^8$ CFU/mL after 18 h were achieved for milk and rice flour fermentations, respectively), lactic acid concentration (maximum values of 1.2 ± 0.3 g/L and 4.4 ± 0.21 g/L were achieved after 24 h of fermentation process for milk and rice flour, respectively) and production of a selected functional metabolite, considered as responsible of the immunomodulatory activity of the postbiotics obtained (6.93 ± 0.5 mg/L and 17.23 ± 0.28 mg/L after 24 h of fermentation, for milk and rice flour, respectively).

An aqueous suspension of cooked beans was fermented without pH control in two different mixing conditions.

The mixing system was improved by designing an impeller that guarantees a higher homogeneity of the medium inside the reactor: the results showed better performances in terms of growth rate and lactic acid production, reaching a microbial charge of approximately 1×10^9 CFU/mL after 14 h and a lactic acid content of 2 g/L after 16 h of process.

The functional peptide was not detected in the fermenting bean suspension, so a further optimization of the protocol will be necessary for this purpose. A thermal treatment at 90°C for 1 min, for rice and milk, and at 90°C for 15 min, for beans, was sufficient to reduce the bacterial charge in the fermented product without negatively affecting the products quality; freeze drying and spray drying technologies had the same impact on the food products, not altering their organoleptic and chemical properties: a promising result since spray drying technology is usually used at larger scale.

The results obtained at pilot scale for milk and rice flour were completely overlapped.

During milk fermentation, a maximum bacterial load of $6.7 \times 10^8 \pm 2.65 \times 10^8$ CFU/mL was reached after 20 h of process; a lactic acid content of 2.1 ± 0.25 g/L and a peptide concentration of 14.20 ± 0.80 mg/L were observed at the end of fermentation. As for rice flour, a maximum bacterial growth of $1.27 \times 10^9 \pm 6.9 \times 10^8$ after 18 h of process, a maximum lactic acid production of 6.03 ± 0.7 g/L after 24 h and a maximum functional peptide content of 22.60 ± 4.10 mg/L after 20 h of fermentation were obtained. For both scales, rice flour fermentation gave better results in terms of bacterial growth, lactic acid and functional peptide production, probably for the rice prebiotic components.

Also, at pilot scale, a thermal treatment of 90°C for 1 min was sufficient to reduce the bacterial charge in the fermented products and it was confirmed that spray drying technology had a low impact on the product. Moreover, a shelf-life analysis of the products obtained at pilot scale was performed at three different storage temperatures (4°C, 20°C and 37°C) for six months to evaluate the food product stability over time: dried fermented rice resulted more stable than milk.

INTRODUCTION

Innovation in food industry consists of combining new scientific and technical approaches in food processing, product formulation, and food quality, with novelties at high cultural and social impact in order to produce food that satisfies the nutritional, personal and social needs of all communities.
Food innovation is considered an important instrument for companies belonging to the food industry to stand out from competitors and to satisfy consumer expectations (Menrad, 2004).
Recently, consumer requirements in the field of food production have changed considerably: foods are no more intended to only satisfy hunger and to provide the necessary nutrients, but also to prevent nutrition-related diseases and to improve physical and mental well-being (Menrad, 2003; Robertfroid, 2000b). This increasing interest towards healthier food and lifestyles has focused the scientific research on functional foods, defined by Functional Food Science in Europe (FUFOSE) as foods that beneficially affect one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and/or a reduction of risk of diseases.
In this regard, functional foods play an important role, as demonstrated by their increasing demand derived from the increasing cost of healthcare, the steady increase of life expectancy, and the desire of older and more vulnerable people for improved quality of their years (Kotilainen et al., 2006; Robertfroid, 2000a, b). Although the most common probiotic functional foods are milk and milk-based products, in recent years the scientific interest towards the development of non-dairy-based functional foods is increasing progressively.
The fermentation of cereals, legumes, fruits and vegetables-based foods for the production of new probiotic products, allows an improvement in the nutritional qualities, a beneficial effect on the human health, and responds to the needs of consumers suffering from lactose intolerance, milk protein allergy and high blood cholesterol concentration (Granato et al., 2010).
A novel category of fermented functional foods is represented by postbiotic products, suitable for categories of people with high pathological vulnerability, such as children, elderly people and pregnant women. Postbiotics consist of inactivated microorganisms and molecules, as enzymes, peptides, lipids, organic acids, carbohydrates, proteins, vitamins, cell wall components, or other complex molecules produced in the final or intermediate stage of the metabolic process of these probiotic bacteria. These products exert a beneficial effect on host health and on disease prevention due to their anti-inflammatory and immunomodulatory effect and could be considered a safer alternative to clinical application for immune-deficient patients or infants due the absence of alive microorganisms.

The purpose of this research is the development of efficient processes for the production of functional postbiotic foods through lactic acid fermentation.

Different mechanisms to shock/kill microorganisms without deactivating the metabolite(s) that contribute to the postbiotic effect of the product were used and the effect of freeze-drying and spray-drying treatments on the resulting functionalized powders was investigated. The substrates used for the production of wet and dried functional semi-finished products on laboratory and pilot scale were skim milk and rice flour. Moreover, fermentation, deactivation and drying processes were performed and optimized on laboratory scale for a cooked bean suspension.

As only few studies have been performed on a leguminous substrate until now and in literature many experimentations are not reported on this kind of fermenting medium, a detailed study of the most appropriate pre-treatment to be applied on the bean suspension to be fermented was carried out.

Chapter 1

1. Functional Foods

"Food as medicine" philosophy is not an entirely new concept. "Let food be your medicine and medicine be your food" was a tenet exposed by Hippocrates in approximately 400 B.C.

Almost 2500 years later the idea of food as an instrument able to beneficially influence some biological functions and reduce the risk of diseases development (Menrad, 2003) is once more gaining importance and it is the basis of the concept of functional foods.

Functional foods fall in the middle area between conventional foods and medicine: they are primarily foods and in food form but their intake offers specific health-enhancing properties (Ramesh et al., 2012). The Institute of Food Technologists defines them as "foods and food components that provide a health benefit beyond basic nutrition. These substances provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impact health benefits". Therefore, they could not be in the form of pill or capsule but just as a normal food form, as specified by the European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE).

The general features of a functional food can be summarised as follows (Ramesh et al., 2012):

- They address specific dietary needs or health conditions and are designed for specific consumer groups;
- They are consumed as part of the regular diet;
- Their physiological functionality is based on bioactive compounds;
- The documentation of their safety and efficacy is essential;
- Any health claims or planned claims are based on scientific research and are consistent with regulation.

Kotilainen et al. (2006), Sloan (2000) and Spence (2006) proposed a classification based on the product characteristics:

- Conventional products, that contain naturally occurring compounds with positive effects on human health;
- Fortified products with additional nutrients, such as fruit juices fortified with vitamin C, vitamin E, folic acid, zinc and calcium;

- Enriched product with additional new nutrients or components not normally found in a particular food, like probiotics or prebiotics;
- Altered products from which a deleterious component has been removed, reduced or replaced by another with beneficial effects, for example fibres as fat releasers in meat or ice cream;
- Enhanced commodities, in which one of the components has been naturally enhanced, e.g., eggs with increased omega-3 content.

An alternative classification based on the aim of functional foods (Makinen-Aakula, 2006) is the following:

- functional foods that add good to life or improve children's life, like prebiotics and probiotics;
- functional foods that reduce an existing health risk problem such as high cholesterol or high blood pressure;
- functional foods which make life easier, such as lactose-free or gluten-free products.

1.1 Functional components and health benefits

Cereals, legumes, fruit and vegetables are common products rich in nutraceutical components.
Table 1.1 reports a summary of the main functional components of foods and their potential benefits (El Sohaimy, 2012). These bioactive substances can be naturally present in the food product, or added, or enhanced in concentration and/or in their biological effect by appropriate processes.
Gut health is increasingly identified as a key factor in several diseases and in general wellbeing.
This has led to much scientific research trying to link health benefits to gut health.
Gut health is determined by the interplay between nutrition, gut microbiota, functional components, and the ways in which these interact with each other and with the host. The beneficial effects provided by gut health enhancing components can be induced in various areas of human health:

- *Immunity*: infections, auto-immune diseases, allergies;
- *Mood*: sleeplessness, depression, stress;
- *Gut health comfort*, preventing bloating, flatulence, and constipation, lactose and gluten intolerance;
- *Metabolic*: obesity, diabetes Mellitus type 2, insulin resistance, cardiovascular diseases, hypertension;
- *Longevity*: osteoporosis, mental health, energy level, longevity and overall well-being.

Table 1.1 Examples of functional food components (El Sohaimy, 2012).

Functional components	***Source***	***Potential benefits***
Carotenoids: α-carotene β-carotene Lutein Lycopene	- Carrots, fruit and vegetables; Green vegetables Tomato products	Neutralize free radicals; Reduce risk of macular degeneration Reduce the risk of prostate cancer,
Dietary fibres: Insoluble B-glucan Soluble	Wheat bran Oat, Barley Psyllium	Reduce risk of breast or colon cancer; Reduce risk of cardiovascular disease; Protect against heart disease and cancers; lower LDL and total cholesterol
Fatty acids: Long chain omega-3 Fatty Acids-DHA/EPA Conjugated Linoleic Acid	Salmon and other fish oils Cheese, meat products	Reduce risk of cardiovascular disease. Improve mental, visual functions
Phenolics Anthocyanidins Catechins Flavanones Flavones Lignans Tannins	Fruits Tea Citrus Fruits/vegetables Flax, rye, vegetables Cranberries, cocoa, chocolate	Neutralize free radicals; reduce risk of cancer; Improve urinary tract health. Reduce risk of cardiovascular disease
Plant Sterols: Stanol ester	Corn, soy, wheat, wood oils	Lower blood cholesterol levels by inhibiting cholesterol absorption
Prebiotics/Probiotics Fructo-oligosaccharides (FOS); Lactobacilli	Jerusalem artichokes, shallots, onion powder Yogurt, Other dairy	Improve quality of intestinal microflora; gastrointestinal health

In particular, short chain fatty acids (SCFAs), as acetate, propionate and butyrate, polyphenols, gamma-aminobutyric acid (GABA), bioactive peptides, serotonin, polyamines, resistant starch, prebiotics, and probiotics are some of the most studied components to achieve gut health (Parker et al., 2020; Filosa et al., 2018). Foods can be directly enriched in some of these functional components or in their specific precursor molecules, which resist the action of digestive enzymes and the adsorption in the upper gastrointestinal apparatus, arrive in the gut and then can be fermented by the gut microbiota, providing the production of health enhancing compounds.

Lactic acid can also be considered a compound with functional properties as, beyond exerting an antimicrobial action, can lead to butyrate production in the human gut by cross-feeding (Bourriaud et al., 2005; Detman et al., 2019; Duncan et al., 2004).

1.2 Prebiotic, Probiotic and Postbiotic functional foods

- Probiotics are live microorganisms, typically beneficial bacteria or yeasts, which confer a health benefit on the host if administered in adequate amounts (Hill et al., 2014).

  The health claims of probiotics are numerous: benefits to the immune system, lowering the hypertension (Gómez-Guzmán et al, 2015), prevention of cancer (Hirayama & Rafter, 2000), reduction of allergic symptoms (Ouwehand, 2007) and cholesterol levels (Bruzzese et al., 2006). The most common types of probiotics are lactic acid bacteria (LABs) (Nichols, 2007), which are normally found in fermented foods. They can enter the host body through food ingestion, survive in the acidic environment of the stomach, and later colonize the gut, where the bacteria will be able to convert carbohydrate into lactic acid and other essential nutrients (Peng et al., 2020).

  These beneficial microorganisms inhabit the gastrointestinal (GI) tract for a longer period of time and provide mutual benefits to the host, including protecting against enteric pathogens, and regulating host immunity (Thursby & Juge, 2017). An important benefit associated with lactic acid production from LABs is the lowering of pH level in the host's gut, which can prevent the growth and survival of pathogenic microbes (Nichols, 2007).

- Prebiotics are defined as selectively fermented ingredients that allow specific changes, both in the composition and activity of the gastrointestinal microbiota that confers benefits upon host wellbeing and health (Gibson et al., 2004). These components resist the hydrolysis of the digestive enzymes and are not absorbed in the upper part of the gastro-intestinal tract.

  When they pass into the large intestine, they are degraded by the indigenous intestinal microbiota. The effect of a prebiotic is essentially indirect because the benefit to the host is mediated through selective stimulation of the growth and/or the activity of one or a limited number of colonic bacteria, thus improving host health (Wang, 2009). Dietary carbohydrates, as fructo-oligosaccharide (FOS), galacto-oligosaccharides (GOS), inulin, isomalto-oligosaccharides (IMO), polydextrose, lactulose, sugar alcohols, and resistant starch are considered as the main prebiotic components. Besides altering gut microbiota, prebiotics can improve gut integrity and function, decrease gut pH, and reduce levels of nitrogenous metabolites (Watson & Preedy, 2015). They can promote a direct SCFA production or they can generate SCFAs indirectly via cross-feeding. Indeed, they could be fermented by several microbes in the gut and the resulting metabolites could be utilized by others (Holscher, 2017) for SCFA production.

- According to Tsilingiri et al. (2013), postbiotics include any substance released or produced through the metabolic activity of microorganisms, which exerts a beneficial effect on the host, directly or indirectly. They consist of inactivated microorganisms and molecules (enzymes, peptides, organic acids) produced in the final or intermediate stage of the metabolic process of these probiotic bacteria. Although postbiotics do not contain live microorganisms, they show a beneficial health effect through similar mechanisms that are characteristic of probiotics, minimizing the risks associated with their intake. Postbiotics have pleiotropic effects, including immunomodulatory, anti-inflammatory, antioxidant, and anti-cancer properties (Zółkiewic et al., 2020). In particular, as these molecules might have a positive influence on the regulation of the physiological functions of the host, they might be a safer option for immune-deficient patients or infants because they do not require the introduction of any live bacteria (Kareem et al., 2014; Cicenia et al., 2014). In most studies, postbiotics derive from a fermented culture medium that is being subsequently filtered or heated, resulting in a liquid cell-free supernatant (CFS) or cell-free spent medium (CFSM) (Zółkiewic et al., 2020). The inactivation of probiotics can be carried out using heat, filtration, sonication, centrifugation, and ultraviolet radiation (UV). In this case, bacterial lysis can occur, releasing a large array of compounds such as DNA, enzymes, lipoteichoic acids, and other intracellular metabolites that can serve as potential postbiotics (Taverniti, 2011). In Table 1.2, some of the main postbiotic compounds, the corresponding probiotic microorganisms and the resulting health benefits are reported.

Table 1.2. Examples of postbiotics obtained from probiotics and their health benefits.

Postbiotic	Examples	Probiotic sources	Biofunction	References
Polypeptides	Bacitracin Nisin Reuterin	*Bacillus subtilis*	Inhibit the growth of competitive microbes; Modulate the intestinal microflora in host	Heng et al., 2007; Perez et al., 2014 Rattanachaikunsopon & Phumkhachorn, 2010; Reis et al., 2012; Šušković et al., 2010
Short chain fatty acids	Acetate, Butyrate, Propionate	*Bifidobacterium*, *Enterococcus*, *Lactobacillus*	Stimulate the growth of beneficial microbes; Modulate electrolytes and water absorption; Regulate host's gut immunity	Al-Sheraji et al., 2013; den Besten et al., 2013; Morrison & Preston, 2016; Nagpal et al., 2018; Peng & Biswas, 2017; Russo et al., 2019
Poly-unsaturated fatty acids	Arachidonic acid, Docosahexaenoic acid, Linoleic acids, Linolenic acids	*Bifidobacterium*, *Lactobacillus*	Interfere with the growth of pathogens; Regulate host's gut immunity; Scavenge excessive free radicals; Suppress the process of carcinogenesis	Andrade et al., 2012; Marion-Letellier, 2015; Peng & Biswas, 2017
Vitamin B	Biotin, cobalamin, folate, niacin, pantothenic acid, pyridoxine, riboflavin, thiamine	*Bifidobacterium*, *Lactobacillus*, *Lactococcus*	Regulate fatty acid metabolism; Influence amino acid metabolism; Promote cell division and growth; Maintain body homeostasis	Capozzi et al., 2012; LeBlanc et al., 2011, 2013; Masuda et al., 2012; Revuelta et al., 2018; Rossi, et al., 2011; Thakur et al., 2016

1.2.1 Milk-based functional foods

Milk and milk products are important constituents of a daily diet, especially for vulnerable groups such as infant's school age children and old age (Donovan, 2006; Gasmalla et al., 2013).

The high content in bioactive components and nutrients, such as specific organic acids, vitamin A, B12, D, riboflavin, calcium, carbohydrates, phosphorous, selenium, magnesium, zinc, proteins, bioactive peptides and oligosaccharides, has attracted the interest of nutritionists, researchers and consumers towards milk-based products for several years.

In addition to nutritional benefits, milk plays a significant role in the control of chronic diseases, such as hypertension, obesity, cancer, osteoporosis, and diabetes (Gasmalla et al., 2017).

In particular, fermented dairy products exhibit a functionality which is directly linked to the action of probiotic microorganisms used as starter culture or which is based on the beneficial properties of microbial metabolites, generated during the fermentation process.

The main health benefits of fermented dairy products are mostly based on the positive effect they exert on the immunity response (Bath & Bath, 2011), their antimicrobial activity, the improvement in lactose metabolism, the reduction in serum cholesterol, their antimutagenic, anticarcinogenic, and anti-diarrheal properties, and the improvement in inflammatory bowel disease (Gotcheva et al. 2002; Nomoto 2005; Shah, 2007; Vijayendra & Gupta 2012).

Lactic acid fermentation is one of the most important food processing technologies to produce probiotic foods, available on the market mainly as dairy-based products consisting of several types of fermented milks, yogurts and cheeses (Gomes & Malcata, 1999).

Moreover, milk-based probiotic powders are produced in order to enhance their long-term stability in terms of viability and functional activity (Carvalho et al., 2003), reducing the transportation and storage costs. In particular, they can be used as an ingredient or supplement of juices, cookies, ice cream, sweets, milk drinks and as powdered milk substitute in recipes, providing nutritional and functional value to the final product (Kim & Bhowmik, 1990; Kumar & Mishra, 2004; Tamime & Robinson, 2007). Furthermore, it was demonstrated that a milk-based postbiotic is able to provide benefits to the immune system of infants (Agostoni et al., 2007), stimulating the innate immunity, providing an anti-inflammatory effect and obviating problems related to probiotic intake, which could potentially be dangerous for an immature infant immune system (Zagato et al., 2014).

1.2.2 Non-dairy based functional foods: cereals and legumes

In recent years, fermentation of cereals, legumes, fruits and vegetables-based foods was investigated for the development of non-dairy based functional food. It not only allows an improvement in the nutritional qualities of the product and a positive effect on human health but can also respond to the needs of consumers suffering from lactose intolerance, milk protein allergy and high blood cholesterol concentration (Granato et al., 2010).

- **Cereals** may be considered optimal fermentable substrates for the growth and the metabolic activity of probiotic microorganisms, especially lactobacilli and bifidobacteria, with a powerful prebiotic effect due to their content in specific non-digestible carbohydrates like oligosaccharides. Their nutritional quality and their sensorial properties are sometimes inferior in comparison with milk or milk-based products due to the lower protein content, the deficiency of certain essential amino acids (lysine), the low starch availability, the presence of determined antinutrients (phytic acid, tannins and polyphenols), and the coarse nature of the grains (Chavan & Kadam, 1989).

  In Table 1.3, the composition of different varieties of cereals is compared to that of milk.

Cereal protein digestibility is lower than that of animal source, due partially to the presence of some antinutrients (Table 1.4), as phytic acid, tannins, and polyphenols which bind to protein and make them indigestible (Oyewole, 1997). Fermentation may be the most simple and economical way of improving their nutritional value and their functional qualities, making them healthy non-dairy carriers to prepare prebiotic, probiotic and postbiotic foods (Prado et al, 2008).

In particular, lactic acid fermentation of different cereals, such as maize, sorghum, finger millet, has been found effectively to reduce the amount of phytic acid, tannins and to improve protein availability (Chavan et al., 1988; Lorri and Svanberg, 1993). An increase in riboflavin, thiamine, niacin, and lysine content owing to the action of lactic acid bacteria was observed in various fermented blends of cereals (Hamad and Fields, 1979; Sanni et al., 1999).

Khetarpaul and Chauhan (1990) reported an improved availability of minerals in pearl millet fermented with pure cultures of lactobacilli and yeasts. Another possible application of cereals or cereal constituents in functional formulations consists of encapsulation of probiotic strains.

In particular, the high amylose content in starch granules can be used as ingredient for probiotic encapsulation, allowing to improve the survival of encapsulated bacteria in comparison with standard formulations without starch (Wang et al., 1999; Sultana et al., 2000).

Table 1.3. Nutritional composition of some cereals in comparison with milk. The composition is expressed on 100 g of edible portion (Charalampopoulos et al., 2002).

Parameter	Malt	Rice	Corn	Wheat	Sorghum	Millet	Milk (liquid)
Water (%)	8	12	13.8	12	11	11.8	87.4
Protein (g)	13.1	7.5	8.9	13.3	11	9.9	3.5
Fat (g)	1.9	1.9	3.9	2.0	3.3	2.9	3.5
Carbohydrates (g)	77.4	77.4	72.2	71.0	73.0	72.9	4.9
Fiber (g)	5.7	0.9	2.0	2.3	1.7	3.2	n.d.
Ash (g)	2.4	1.2	1.2	1.7	1.7	2.5	0.7
Ca (mg)	40	32	22	41	28	20	118
P (mg)	330	221	268	372	287	311	93
Fe (mg)	4.0	1.6	2.1	3.3	4.4	68	Trace
K (mg)	400	214	284	370	350	430	144
Thiamin (mg)	0.49	0.34	0.37	0.55	0.38	0.73	0.03
Riboflavin (mg)	0.31	0.05	0.12	0.12	0.15	0.38	0.17
Niacin (mg)	900	1.7	2.2	4.3	3.9	2.3	0.1
Mg (mg)	140	88	147	113	n.d.	162	13

Table 1.4. Main antinutrients present in several common cereals and their composition.

Cereal	*Antinutrients*	*Content*	*Reference*
Maize	Phytic acid Polyphenols	87.6 ÷ 682.20 mg/100 g 363.71÷706.15 mg/100 g	*Sokrab et al., 2011*
Wheat	Tannins Phytic acid	1,43÷1,84 mg/g $_{db}$ 7.95÷8.00 mg/g $_{db}$	*Singh et al. 2012*
Rice	Phytic acid Polyphenols	93.7 mg/100 g 172.11 mg/100 g	*Kaushal et al., 2012*
Pearl millet	Tannins Phytic acid	0.459 mg/100 g 5.00 mg/100 g	*Singh et al., 2017*
Finger millet	Tannins Phytic acid	0.301 mg/100 g 8.6 mg/100 g	
Sorghum	Tannins Phytic acid	0.601 mg/100 g 3.4 mg/100 g	

- **Legumes** are another interesting alternative to milk-based functional foods.

  Legumes, nuts, seeds and soy represent an important source of dietary vegetable protein intake and a desirable alternative to protein foods from animal origin, for reasons related to human health, environmental impact and eating habits. The scientific interest towards the development of legume-based functional foods is increasing progressively: legumes provide high protein meat-substitutes for vegetarians, low fat substitutes for health-conscious individuals and low-cost products for low-income groups (Maphosa & Jideani, 2017). Moreover, legumes are gluten free and therefore suitable for consumption by celiac disease patients. Their low glycaemic index (GI) rating for blood glucose control (Khalid & Elharadallou, 2013) makes them suitable for diabetic patients. As shown in Figure 1, legumes contain higher amounts of protein and dietary fibre than cereals. They are an excellent source of 20–45% good quality proteins, generally rich in the essential amino acid lysine. Leguminous proteins, except soy protein, are low in the essential sulphur-containing amino acids (SCAA), methionine, cystine, cysteine, and tryptophan and, for this reason, they can be considered an incomplete source of proteins (Kouris-Blazos & Belski, 2016). However, the low level of SCAA in legumes is not completely a negative factor as it provides an increased calcium retention in comparison with protein from animal or cereal origin. In Table 1.5, a comparison between rice flour and white bean composition in terms of vitamins, minerals, and aminoacids is reported, confirming their complementarity in terms of nutrients provided: a nutritional balance can be reached when legumes are eaten in combination with cereals (FAO, 2016).

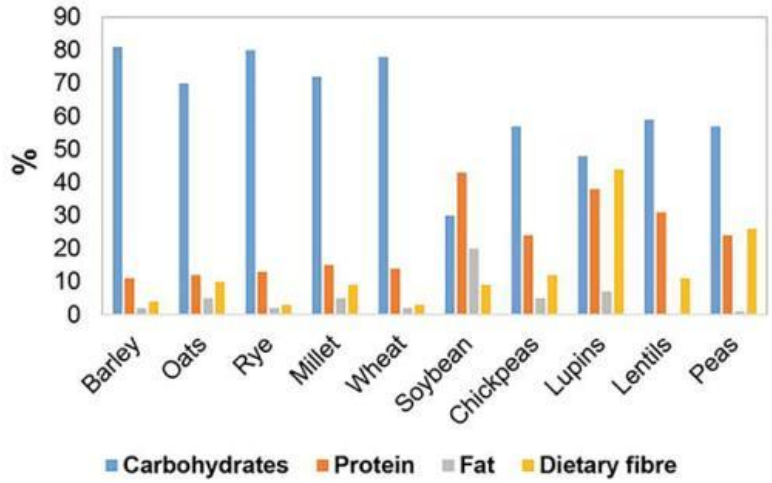

Figure 1.1. A comparison between some common cereal grains and legumes in terms of nutritional composition (Maphosa & Jideani, 2017).

Fermentation process on leguminous substrates can influence the presence of various bioactive components, such as vitamins, natural phenolics and bioactive peptides (Gan et al., 2017) and can promote the reduction of anti-nutritional factors, as oligosaccharides, allergenic proteins, proteinase inhibitors, trypsin inhibitors, saponins, and phytates, that limit their consumption and affect the digestibility and the bioavailability of nutrients (Worku & Sahu, 2017).

These anti-nutrient compounds are non-toxic, but generate adverse physiological effects on human health, interfering with protein digestibility and bioavailability of some minerals (Sanchez-Chino et al., 2015). In Table 1.6, the composition in some antinutritional compounds of common legumes is reported. Most of these non-nutrients are phytochemicals with antioxidant properties and a key role in the prevention of some cancers, heart diseases, osteoporosis and other chronic degenerative diseases (Bouchena & Lamri-Senhadji, 2013; Messina, 2016).

Legumes also contain significant amounts of resistant starch and oligosaccharides, mainly α-galactosides, as raffinose, stachyose and verbascose, which have been reported to possess prebiotic properties (Kouris-Blazos & Belski, 2016). They are fermented by probiotics to short chain fatty acids, promoting the maintenance of colonic health. On the other hand, these sugars are responsible for several discomforts, such as bloating, flatulence and constipation, since they pass undigested to the colon and form large amounts of carbon dioxide, hydrogen and methane, when metabolised by bacteria. Lactic acid fermentation process could be a strategy to reduce α-galactosides content in legumes, making them more digestible and mitigating the discomfort linked to their consumption (Granito et al., 2006; Battistini et al., 2018; Kitum et al., 2020).

Moreover, it was reported that the lactic fermentation of adzuki beans and lentils allowed the accumulation of γ-aminobutyric acid (GABA), a blood pressure regulator (Liao et al, 2013; Torino et al, 2013) while the cowpeas fermentation with *Lactobacillus plantarum* led to changes in phenolic composition and improved antioxidant activity (Dueñas et al., 2005).

Table 1.5. Comparison between rice flour and white bean in terms of vitamin, mineral and amino acid content, referred to 150 g of product (www.nutritionvalue.org).

		Beans [mg]	**Rice flour** [mg]			**Beans** [mg]	**Rice flour** [mg]
Minerals	*Calcium*	109.5	15	***Amino-acids***	*Alanine*	564	498
	Iron	4.26	0.525		*Arginine*	832	774
	Magnesium	102	52.5		*Asparagine*	-	-
	Potassium	694.5	114		*Aspartic Acid*	1627	823.5
	Cupper	0.223	0.195		*Cysteine*	147	160.5
	Manganese	0.765	1.8		*Glutamic acid*	2052	1645.5
	Selenium	0.002	0.022		*Glycine*	525	400.5
	Zinc	1.635	1.2		*Histidine*	375	223.5
	Phosphorus	253.5	147		*Isoleucine*	594	366
	Sodium	3	-		*Lysine*	924	310.5
Vitamins	*Vit B6*	0.19	8.7		*Methionine*	52,5	216
	Vit B12	0	0.207		*Phenylalanine*	727	475.5
	Folate (B9)	0.201	0.031		*Proline*	570	417
	Thiamine (B1)	0.354	3.885		*Serine*	732	465
	Riboflavin(B2)	0.045	1.228		*Threonine*	565	315
	Niacin (B3)	0.408	0.654		*Tryptophan*	159	108
	Vit A	0	0.006		*Tyrosine*	379	471
	Vit C	0	0		*Valine*	703	522
	Pantothenic Acid	0.38	0.165		*Leucine*	1074	732
					Glutamine	-	-

Table 1.6. Composition of main antinutrients present in several common legumes (Amarowicz & Pegg, 2008; Gulewicz et al., 2014).

Legumes	**Polyphenols (%)**	**Phytic acid (%)**	**Tannins (%)**	**α-Galactosides (%)**
Common bean (white)	0.3	1.0	0	3.1
Common bean (brown)	1.0	1.1	0.5	3.0
Pea	0.2	0.9	0.1	5.9
Lentils	0.8	0.6	0.1	3.5
Faba bean	0.8	1.0	0.5	2.9
Chickpea	0.5	0.5	0	3.8
Soybean	0.4	1.0	0.1	4.0
Pigeon pea	0.2	0.1	0	0

Chapter 2

2 Processes for the production of probiotics and postbiotics

The aim of this chapter will be to provide theoretical principles at the base of the main processes used for the production of wet and dry probiotic or postbiotic functional foods.
The main stages that allow to obtain probiotic or postbiotic products are the following:

1) Appropriate pre-treatment of the food matrix to be processed;
2) fermentation processes carried out on food matrices using selected microorganisms as starter cultures;
3) mild heat treatments (max 80-85°C) can be performed to “turn off" microorganisms without denaturing the functional metabolites produced;
4) a drying process performed on the fermented and/or deactivated substrates can allow to obtain final functional powders with probiotic and/or postbiotic properties.

2.1 Fermentation process

Fermentation consists of a process in which the controlled action of selected microorganisms alters the texture of foods, produces subtle flavours and aromas, preserves foods by production of acids or alcohol, and increases the quality and the value of raw materials.
A fermentation is initiated by inoculating a suitably pre-treated and sterilized substrate with a desired microorganism. The inoculated medium is held under environmental conditions that favour its conversion to the desired products. The fermented substrate can be directly used or further processed, in order to ensure the desired chemical-physical characteristics of the final product.
A bioprocess is typically made up of three steps, as shown in Figure 2.1 (Chisti & Moo-Young, 1999). The raw material or feedstock must be converted to a form which is suitable for processing.
For this purpose, several preliminary operations can be carried out, such as milling, sieving, hydrolysis, medium formulation and sterilization. The second step consists of the bioreaction section, where the desired biotransformation take place; it is followed by a downstream processing stage, where physical operations are aimed at concentration, purification, or treatment of the final product for specific different applications (Chisti & Moo-Young, 1999).

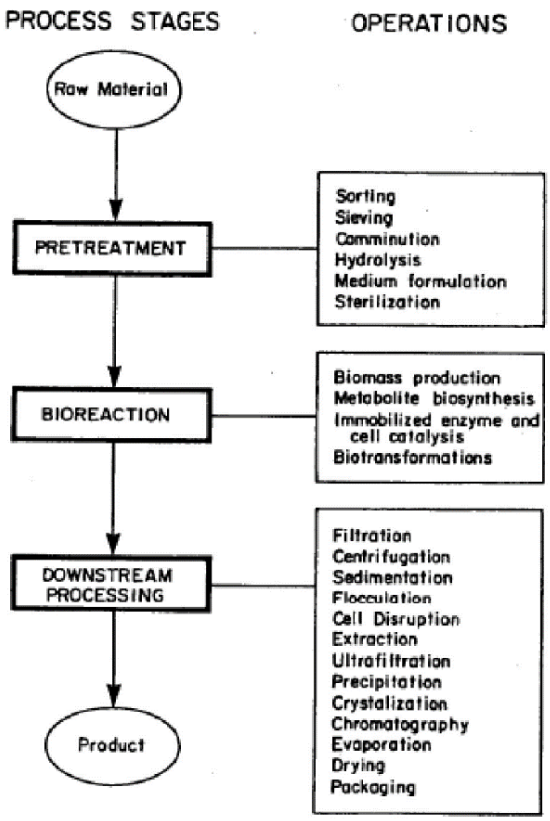

Figure 2.1. Bioprocess stages and the commonly used operations in them (Chisti & Moo-Young, 1999).

Typically, fermentation is a natural process. Spontaneous fermentation may fail, because it is not possible to ensure the same quality of the end product. For this reason, the control of the process is extremely important: in fact, the choice of the starter cultures and thus, the selection of the optimal operating conditions that correspond to the best growth conditions of the microorganism, have a critical impact on the palatability, processability, nutritional attributes, sustainability, and safety of food. Due to a wide variety of possible substrates, microorganisms, and products, fermentation processes can be extremely diverse. Examples of different products obtained by fermentation include bread, cheese, wine, beer, coffee, medicinal and industrial enzymes, amino acids, antibiotics, soy sauce, compost, biopolymers, bioplastics, microbial oils, flavours, colorants, specialty chemicals, vaccines, therapeutic proteins, and numerous other products (Fellows, 2000).

The main advantages of fermentation as a method of food processing are:

- the use of mild conditions of pH and temperature which allows to maintain and often improve the nutritional and sensory properties of the food;
- the production of particular flavours or textures that cannot be achieved by other methods;
- low energy consumption due to the mild operating conditions;
- relatively low capital and operating costs;

- relatively simple technologies.

The main factors that control the growth and activity of microorganisms in food fermentations are:

- availability of carbon and nitrogen sources, and any specific nutrient required by individual microorganisms;
- pH and incubation temperature;
- moisture content;
- redox potential;
- stage of microorganism growth;
- presence of other competing microorganisms.

2.1.1 Modes of Fermentation

There are three main modes of fermentation technique: batch, fed-batch and continuous (Figure 2.2). Most industrial fermentation processes operate as simple batch or fed-batch; continuous fermentation is relatively rare. There are few continuous fermentation examples in industry, for example vitamin C, propionic acid and Quorn production or the treatment of wastewater by activated sludge method, while brewing of beer and most antibiotic fermentations are conducted in batch or fed - batch modes of operation (Macauley-Patrick & Finn, 2008; Chisti, 2010).

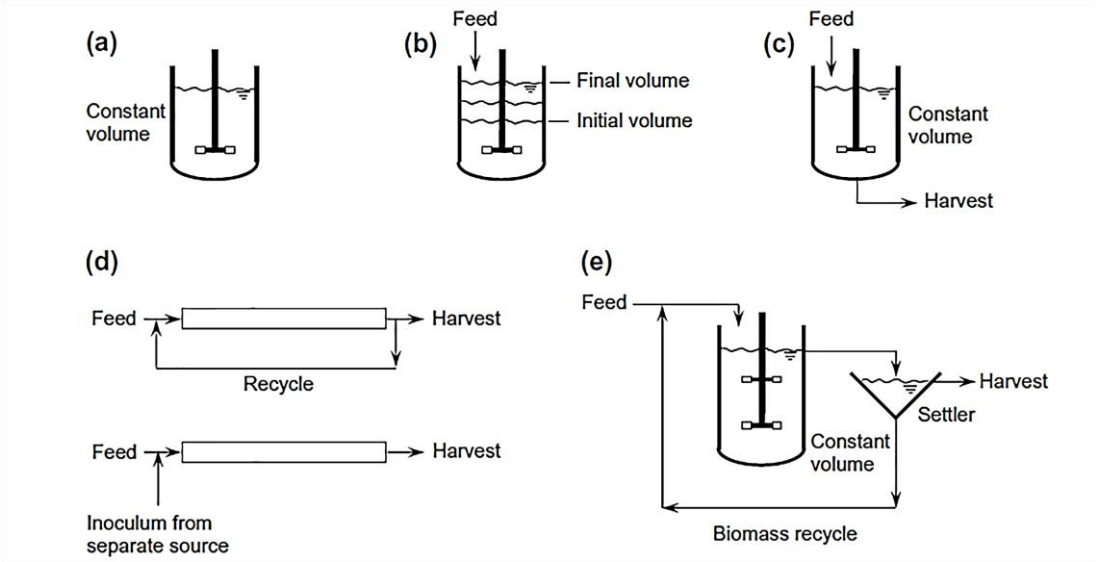

Figure 2.2. Fermentation methodologies. (a) Batch fermentation; (b) fed-batch culture; (c) continuous flow well mixed fermentation; (d) continuous plug flow fermentation with and without recycle; (e) well - mixed continuous fermentation with recycle of concentrated biomass (Chisti, 2010).

- In batch fermentation (Figure 2.2a), a batch of culture medium in the fermenter is inoculated with a microbial culture, called "starter culture". The process is carried out for a certain duration ("batch time" or "fermentation time") and the product is harvested.

  Since fresh media is not added during the incubation period, concentration of nutrients decreases continuously, the biomass increases and various toxic metabolites also accumulate in the culture vessel together with the biomass and the desirable metabolites. Therefore, batch culture technique gives characteristic growth curves with lag phase, log phase, stationary phase, and decline phase. A scheme of a simple batch configuration is shown in Figure 2.3.

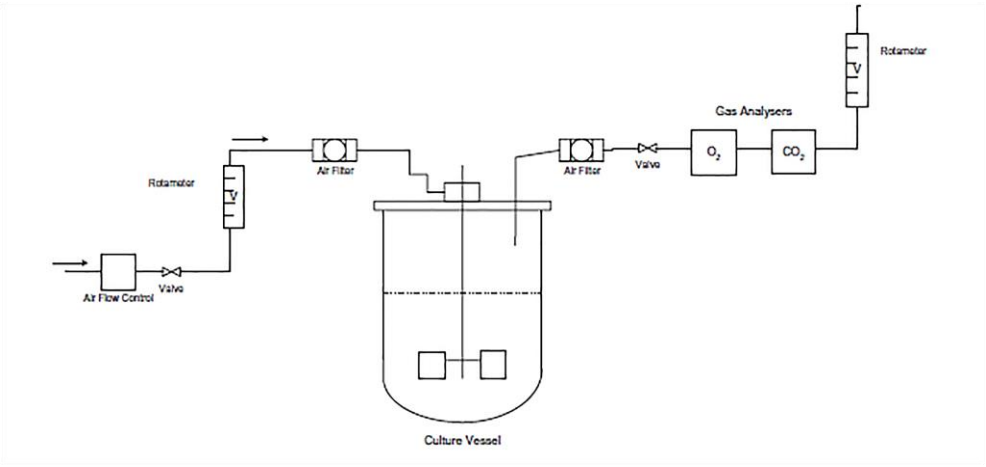

Figure 2.3. A diagram of a simple batch fermentation (Macauley-Patrick & Finn, 2008).

- In fed-batch fermentations, sterile culture medium is added either continuously or periodically to the fermenter (Figure 2.2b). The volume of the fermenting broth increases with each addition of the medium and the composition of the feeding medium may vary with time. The batch is harvested after the batch time. This feeding strategy allows the microorganism to grow at the desired specific growth rate, minimising the production of undesirable by-products, and allowing the achievement of high biomass and product concentrations (Macauley-Patrick & Finn, 2008). The addition of a certain quantity of nutrients during the non-exponential growth phase increases the production of secondary metabolites. A simple fed-batch fermentation set-up is represented in Figure 2.4.
- In continuous fermentations, sterile medium is fed continuously into a fermenter and the fermented product is continuously withdrawn, so the fermentation volume remains unchanged (Figure 2.2c). Typically, continuous fermentations are started as batch cultures and the continuous feeding begins after the microbial population has reached a certain concentration.

  In some continuous fermentations, a small part of harvested culture may be recycled, to continuously inoculate the sterile feed medium entering the bioreactor (Figure 2.2d).

Moreover, there are well-mixed continuous fermentations where a part of the biomass in the harvest stream is concentrated and recycled to the fermenter (Figure 2.2e). They are used commonly in wastewater treatment by activated sludge method (Chisti, 2010). In Figure 2.5, a continuous fermentation configuration is reported. In Table 2.1 the main advantages and disadvantages of each configuration are summarized.

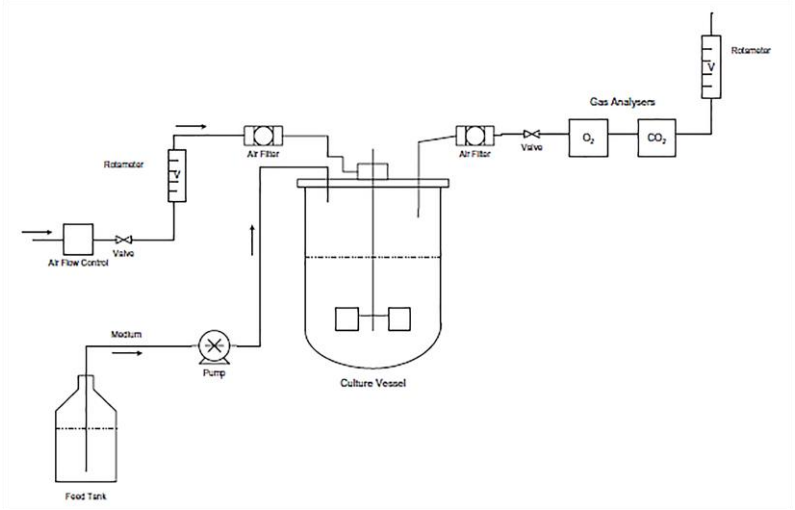

Figure 2.4. A simple fed-batch fermentation set-up (Macauley-Patrick & Finn, 2008).

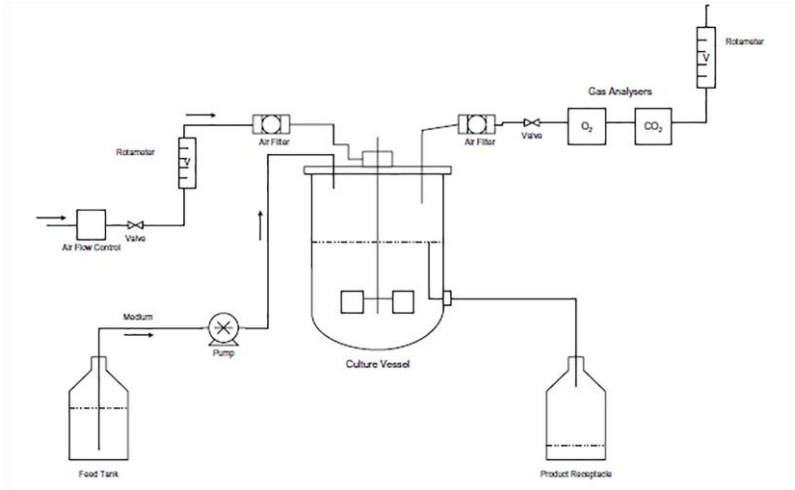

Figure 2.5. A continuous fermentation set-up (Macauley-Patrick & Finn, 2008).

Table 2.1. Main advantages and disadvantages of three possible modes to conduct the fermentation process: batch, fed-batch and continuous methods (Macauley-Patrick & Finn, 2008).

Modes	Advantages	Disadvantages
Batch	•Simplicity of use: batch culture can be easily readied, and can be carried out in less than 24 h; • operability and reliability: less likely to have instrument failure on short batch runs; • production of secondary metabolites not growth-related (i.e., produced when the microorganism is in stationary phase); • fewer possibilities of contamination; • easy to assign a unique batch number to each run, generating high confidence in the history of each batch of product.	• Culture ageing can affect the production of growth-related products; • build-up of toxic metabolites can affect cell growth and product formation; • initial substrate concentrations may be limiting due to inhibition and repression effects; • batch-to-batch variability; • presence of a non-productive period owing to cleaning, resterilization, filling and cooling of equipment; • recovery and re-feeding of the microorganism from a bioprocess to another one can lead to degeneration or differentiation; • cellular autolysis during the decline and stationary phase affects the amount of product and its composition;
Fed-batch	• Controlling the concentration of the limiting substrate prevents substrate- inhibition effects and avoids catabolite repression; • by a careful feeding strategy, microorganism growth rate and the oxygen demand are controlled; • high cell density can be achieved by using fed-batch compared to batch culture. Batch culture limits the final cell growth due to nutrient deficiency; • Increased production of non-growth-related secondary metabolites; • reduction of broth viscosity owing to fresh medium addition (better agitation and aeration);	•Detailed knowledge of the microorganism growth and product formation pattern is required; • deficiency of reliable online sensors for accurate substrate determination in near real time; • without feedback control, mismatches between feed rates and culture metabolism can occur, leading substrate levels to become depleted or rise to undesirable levels; • the process operators must be fully trained and highly skilled; • contamination can occur more easily.
Continuous	• Productivity and growth rate can be optimized by changing the feed flow rate; • longer periods of productivity with less down time; • maintenance of high cell concentrations in the bioreactor at low substrate concentrations: fresh nutrients are added to the system, products and waste are removed via a membrane; the cells are recycled into the reactor; • the effects of environmental or physical factors can be more easily analysed: any changes in the constant steady state can be attributed solely to the change in those factors.	• The US Food and Drug Administration (FDA) does not accept continuous culture in the production of therapeutic products: such products must be produced into batches for traceability purposes; • some fermented foods and beverages require cellular products released from different phases of batch culture growth for full flavour development. (non-growth-linked products such as antibiotics, monoclonal antibodies and toxins); • contamination can be a major problem in continuous cultivation, resulting in the wash out of the desired organism and a loss of product; • culture mutation can easily occur in continuous processes.

2.1.2 Growth patterns and kinetics in batch cultures

When cells grow in a batch culture, they will typically proceed through a number of distinct phases (Figure 2.6):

1) The lag phase consists of an adaptation period in a new environment, after inoculation is performed. The duration of the lag phase depends on the growth history of the inoculum, the composition of the medium, and the amount of culture used for inoculation (Chisti & Moo-Young, 1999). An excessively long lag phase is undesirable because it penalizes the process productivity; therefore, it should be minimized. Short lag phases occur when the concentration of the medium and the environmental conditions in the seed culture and production vessel are identical (hence less time is needed for adaptation), the dilution shock is small (i.e., a large amount of inoculum is used), and the cells in the inoculum are in the late exponential phase of growth.
2) Once the cells have adapted to the new growth conditions, the exponential or log phase starts. The growth rate of the cells rapidly increases, showing an exponential trend. During this phase, growth-linked metabolites are produced. Nutrient depletion and the formation of inhibitors have the effect of decelerating cell growth. Non-growth-associated products (e.g., classic secondary metabolites) have a negligible rate of formation during this stage while start to be produced as the cells enter the stationary phase (Macauley-Patrick & Finn, 2008).

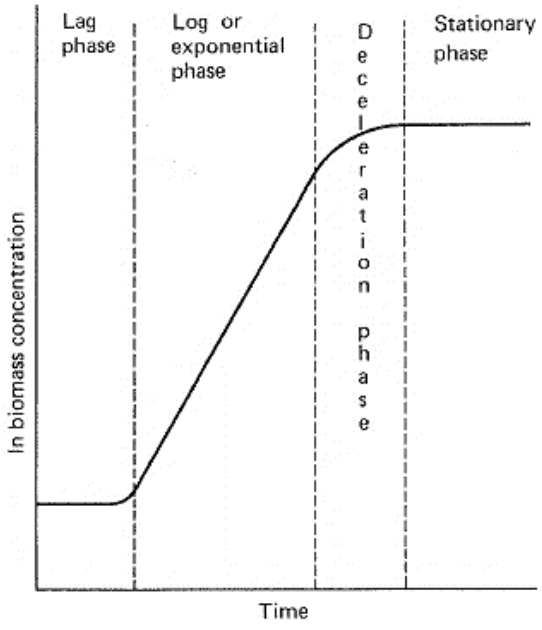

Figure 2.6. Growth of a typical microbial culture in batch conditions (Stanbury et al., 2003).

During the log phase, each cell division results in a doubling of the cell number: after n divisions, the number of cells is 2^n (Figure 2.7). Doubling time, or generation time, t_d, is defined as the time required for the biomass to be doubled. It can be estimated by the Equation 1, assuming a constant maximum value for the specific growth rate, μ_{max}:

$$t_d = \frac{ln\,2}{\mu_{max}} = \frac{0.693}{\mu_{max}} \quad (1)$$

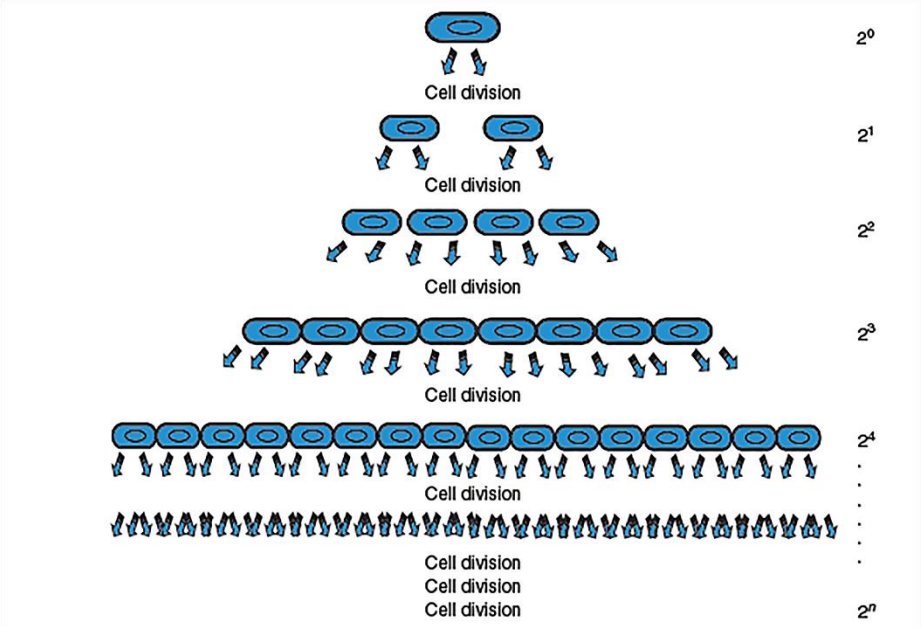

Figure 2.7. Cell division by doubling at each cell generation (Maier, 2009).

During the exponential phase, cell growth in batch conditions can be described by the mass balance reported in Equation (2):

$$\frac{dX}{dt} = \mu X \quad (2)$$

where X is the concentration of cells (mass/volume), t is time, and μ is the specific growth rate constant (1/time).

3) The stationary phase in a batch culture can be defined as a state of no net growth: cells still grow and divide, their biosynthetic activity has not ceased but the slow growth is simply balanced by an equal number of cells dying, according to Equation (3):

$$\frac{dX}{dt} = 0 \quad (3)$$

In this phase, microorganisms have to survive in stress nutrient conditions with high content of toxic and inhibiting metabolites which have accumulated in the fermenting medium; therefore, a slow growth rate is balanced with cell death.

4) The final phase of the growth curve is the death or decline phase. The rate of cell growth decreases to zero while the rate of cell death increases as a result of adverse physical conditions: hence the cell mass reduces. The death phase is often exponential, with a rate of cell death usually slower than the rate of growth during the exponential phase. It can be described by the following Equation (4):

$$\frac{dX}{dt} = -k_d X \tag{4}$$

where k_d is the specific death rate (1/time).

The rate of increase in live biomass concentration when all the nutritional and environmental requirements are provided is proportional to the viable biomass concentration, according to Equation (2). The specific growth rate can be expressed in two different ways: (i) substrate dependent and (ii) substrate independent. One of the first and simplest mathematical model used to describe the specific growth rate as function of substrate concentration is the **Monod model** (Monod, 1949), reported in the following Equation (5):

$$\mu(S) = \mu_{max} \frac{S}{S + K_S} \tag{5}$$

where μ, μ_{max}, S and K_s, are the specific growth rate (1/time), the maximum specific growth rate (1/time), the substrate concentration (mass/volume), the semi-saturation or affinity constant (mass/volume), respectively. In particular, K_s represents numerically the substrate concentration at which growth occurs at one half the value of μ_{max}, as shown in Figure 2.8.

μ_{max} and K_s reflect: (i) the intrinsic properties of the microorganism; (ii) the limiting substrate; (iii) the temperature of growth. As observed in Equation (5), in Monod expression no nutrients other than the limiting substrate and no toxic by-products of metabolism build up are considered.

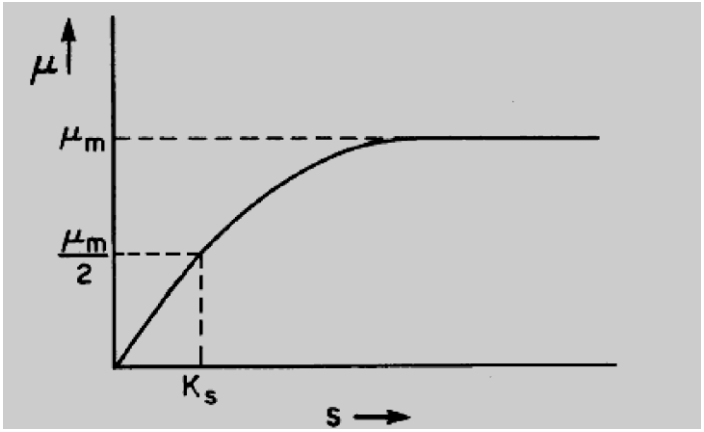

Figure 2.8. Effect of substrate concentration on specific growth rate μ, according to the Monod model. K_s is the substrate concentration at which μ is one half the maximum specific growth rate, μ_{max} (Chisti & Moo-Yang, 1999).

Monod equation has two limiting cases:

- high substrate concentration, when S >> Ks. In this case, the specific growth rate μ is equal to μ_{max}. This simplifies Equation (2) in the resulting relationship of zero order, independent of substrate concentration, shown in Equation (6).

  For S >> Ks: $\frac{dX}{dt} = \mu_{max} X$ (6)

- low substrate concentration, when S << Ks. In this case, growth rate has a first order dependence on substrate concentration, according to Equation (7):

  For S << Ks: $\frac{dX}{dt} = \frac{\mu_{max} S X}{K_S}$ (7)

In Figure 2.9, the specific growth rate as function of the substrate concentration in the two limiting cases of the Monod model is reported.

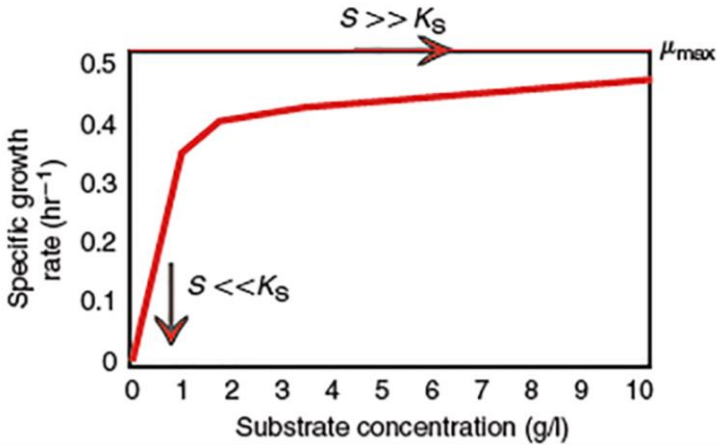

Figure 2.9. Dependence of specific growth rate, μ, on substrate concentration, according to the Monod model. Two limiting behaviours are highlighted: when S >> Ks, μ approaches μ_{max} and becomes independent of substrate concentration; when S << Ks, the specific growth rate exhibits a first-order dependence on substrate (Maier, 2009).

There are several other mathematical models able to describe the growth kinetic during a fermentation. One of them is the **Logistic model**, where the specific growth rate is independent of substrate and is only a function of the biomass concentration in the fermenting medium (Ricker, 1979), according to Equation (8):

$$\mu(X) = \mu_{max} \left(1 - \frac{X}{X_m}\right) \quad (8)$$

where μ_{max}, X and X_m are the maximum specific growth rate (1/time), the biomass concentration (mass/volume) and the maximum biomass concentration (mass/volume), respectively. The logistic equation is useful in processes where information on the limiting carbon substrate is not available.

In the **Contois model**, reported in Equation (9), the growth rate is described as function of both biomass and substrate amounts (Contois, 1959):

$$\mu\,(X, S) = \mu_{max}\,\frac{S}{S + K_s X} \tag{9}$$

where the term (Ks X) can be seen as an apparent Monod constant, proportional to biomass concentration, X.

Moreover, cell growth can be inhibited by various factors and several models have been proposed to describe mathematically this phenomenon:

The **Haldane model**, reported in Equation (10), describes the bacterial growth when inhibition is caused by substrate concentration (Altiok et al., 2006).

Furthermore, Equation (11) reports a dependence of the specific growth rate on the product too, that can exert an inhibiting action on the bacterial growth over certain quantities (Powell, 1984; Pinelli et al, 1997).

$$\mu\,(S) = \mu_{max}\,\frac{S}{S + K_s + (S^2/K_I)} \tag{10}$$

$$\mu\,(S, P) = \mu_{max}\,\frac{S}{S + K_S}\,\frac{k_p}{k_p + P} \tag{11}$$

where μ_{max},S, K_S, K_I, k_p are the maximum specific growth rate (1/time), the substrate concentration (mass/volume), the semi-saturation constant (mass/volume), the substrate inhibition constant (mass/volume) and the product inhibition constant (mass/volume), respectively.

In particular, when $K_I >> S^2$ in the Haldane model, the process is not affected by a substrate inhibition effect and the Equation (10) simplifies in the classical Monod equation; instead, if $K_I << S^2$, the limiting nutrient is responsible of a growth inhibiting effect. Moreover, in Equation (11), k_p represents the maximum concentration of product over which the microbial growth becomes affected by product inhibition. The metabolites produced can be classified based on their production kinetics as specified in Equations (12), (13), (14):

Growth-associated: $r_p = \alpha\, r_x$ (12)

Non-growth-associated: $r_p = \beta\, X$ (13)

Mixed kinetics: $r_p = \alpha\, r_x + \beta\, X$ (14)

where r_p is the production rate (1/time), X is the biomass concentration (mass/volume), and α (mass/mass) and β (mass/mass*time) are the growth linked and non-growth linked constants.

In particular, Equation (14) represents the Luedeking–Piret model for product formation (Luedeking & Piret, 1959a, b).

Moreover, Equation (15) is an example of inhibition of product formation rate by the product concentration itself (Mavituna & Sinclair, 2008):

$$r_p = (\alpha\, r_x + \beta\, X)\,(1 - \frac{P}{P_m}) \quad (15)$$

where P and P_m are the product concentration and the maximum attainable product concentration under inhibition conditions.

2.1.3 Bioreactor design

A bioreactor or fermenter is a special equipment designed in such a way to provide the optimum environment or conditions for growth of high concentration of microorganisms.
In particular, a bioreactor should be capable of the following as a minimum:

- aseptic production for extended period of time;
- meeting the local containment regulations;
- monitoring and controlling pH by either acid/base addition or CO_2/base addition;
- adequate mixing such that the culture remains in suspension, the dissolved oxygen (DO_2) level is maintained and no damage to the microorganisms occurs;
- temperature regulation;
- sterile sampling capability.

The sizes of a bioreactor can vary over several orders of magnitude: the microbial cell (few mm^3), shake flask (100 ÷1000 mL), laboratory fermenter (1 ÷ 50 L), pilot scale (0.3 ÷ 10 m^3) and plant scale (2 ÷ 500 m^3) are all examples of bioreactors.
The design and mode of operation of a fermenter mainly depends on the involved microorganism, the optimal operating conditions required for target product formation, the product value and the scale of production. The design also takes into consideration the capital investments and running costs.
The most common type of bioreactor in use is the STR (stirred tank reactor).
This essentially consists of a vessel with an aspect ratio, i.e., the ratio between its height and its diameter, of around 3:1, and a mixing system typically driven through the headplate, although with some steam in-place systems the mixing will be driven though the base.
The head plate is equipped with ports that allow for the addition of probes, reagents and gas as well as the removal of samples.
There are several alternatives to STR, such as bubble columns or tower fermenters, airlift fermenters, fluidized fermenters, and trickle-bed fermenters, schematized in Figure 2.10.

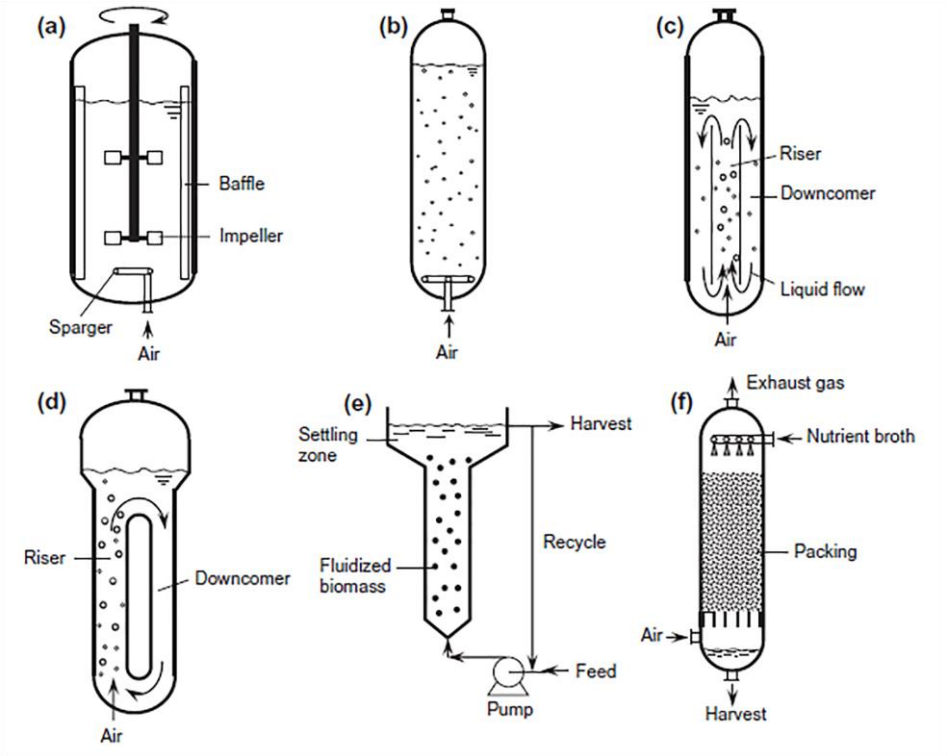

Figure 2.10. Different types of bioreactors for submerged cultures. (a) Stirred tank fermenter; (b) bubble column; (c) internal - loop airlift fermenter; (d) external - loop airlift fermenter; (e) fluidized bed fermenter; (f) trickle - bed fermenter (Chisti, 2010).

The main parts of a fermenter and their function are briefly described below.

The material used for a fermenter. It must have the following properties:

(a) it should not be corrosive; (b) it should not add any toxic substances to the fermenting broth; (c) it should tolerate the steam sterilization process; (d) it should be able to tolerate high pressure and resist pH changes.

Vessel. Small scale vessels with a working volume < 5 litres are constructed of borosilicate glass with single or double stainless steel end plates, where it is possible to easily view the contents.

They are also more economical on medium usage. Using small fermenters with <1 litre working volume, the size and number of samples needed for process monitoring will be limited.

Moreover, small scale vessels are usually sterilised in an autoclave while pilot and industrial scale fermenters are stainless steel systems which require in situ steam sterilisation, thus removing the need for a large on-site autoclave. Vessel can be equipped with a jacket through which steam (for sterilization) or cooling water (for temperature control) is provided (Figure 2.11).

Insufficient heat transfer takes place through the jacket in large scale fermenters: in this case, internal coils are provided in the jacket in order to maintain a constant temperature during the fermentation process.

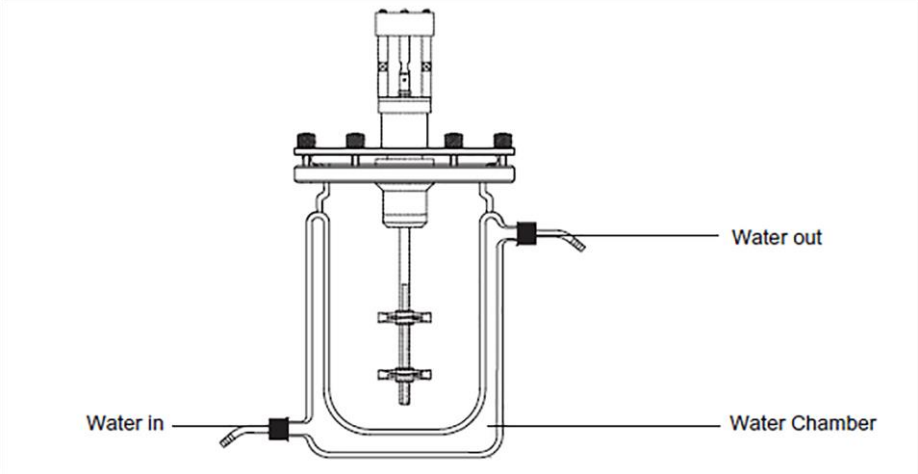

Figure 2.11. Typical small-scale water jacketed glass bioreactor (Matthews, 2008).

Impellers are agitation devices mounted on the shaft and introduced in the fermenter through its lid. They are made up of impeller blades attached to a motor on the lid.

The function of an impeller consists of maintaining a uniform environment in the vessel in terms of heat, nutrient, and, if present, solid particle distribution, fluid and gas mixing, and air/oxygen dispersion. All these things must be achieved efficiently, but without damage to the cells being grown. Impellers also help in breaking foam bubbles in the head space of the fermenter.

The foam formed during the fermentation process must be controlled by the use of appropriate impellers as high foaming may cause contamination in the batch.

The choice of an impeller is dictated by the physical robustness of the cells, the need for oxygen transfer, and by the physical properties of the medium to be fermented. Bioreactors used for microbial cultivation are generally equipped with one or multiple Rushton-type disk impellers, which, together with baffles, provide turbulent flow and efficient dispersion of gas.

A significant increase in mixing time is a drawback of installing multiple Rushton turbines: a combination of Rushton with axial stirrers can reduce the mixing time.

Moreover, the introduction of flow axial components is important when dip-pipes for the addition of substrates and/or pH-controlling reagents to the broth are present: in this case, axial stirrers not only improve the mixing time but avoid pockets of high concentration in the broth.

Cell-culture bioreactors are commonly equipped with marine propellers when a gentler mixing is necessary. Compared to turbines, the diameter of a propeller is generally smaller (one-third the vessel diameter) but nevertheless provides efficient mixing of solids at low power intake.
In Figure 2.12, the most common impellers used in bioreactors are reported. An indication for the power requirement of a mixing system is the dimensionless power number N_P, which is defined by the fluid density, rotational speed, and diameter according to Equation (16).

$$N_P = \frac{P}{\rho\, n^3 D^5} \tag{16}$$

where P is the power, ρ is the fluid density, n is the rotational speed, and D is the diameter of the stirrer.

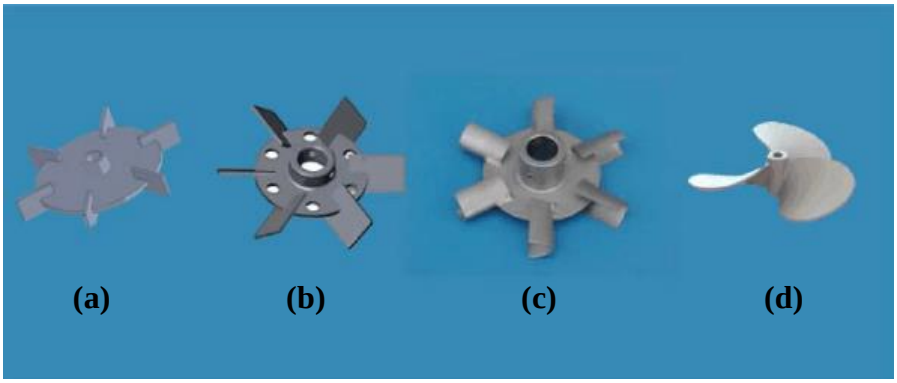

Figure 2.12. Commonly used stirring devices: (a) Rushton, (b) pitched blade, (c) Smith turbine, and (d) marine propeller (Meyer et al., 2017).

Baffles (Figure 2.13) are flat paddle-like structures, typically 10% of the vessel internal diameter. The number of baffles installed varies between 4 and 6. Baffles are placed inside the vessel next to the wall and are used to break the rotational flow of liquid in the vessel that may occur at high agitation rates. The use of baffles eliminates laminar flow, creates a turbulent flow and enhances mass transfer. Moreover, they are used to prevent vortex formation and to improve aeration efficiency.

Figure 2.13. Baffle (Matthews, 2008).

Inoculation Port is the part of the fermenter through which media, substrate and inoculum are added in the fermenter vessel. All inoculations and media transfer should be done aseptically. In Figure 2.14 single, triple and septa methods of introducing fluids are shown.

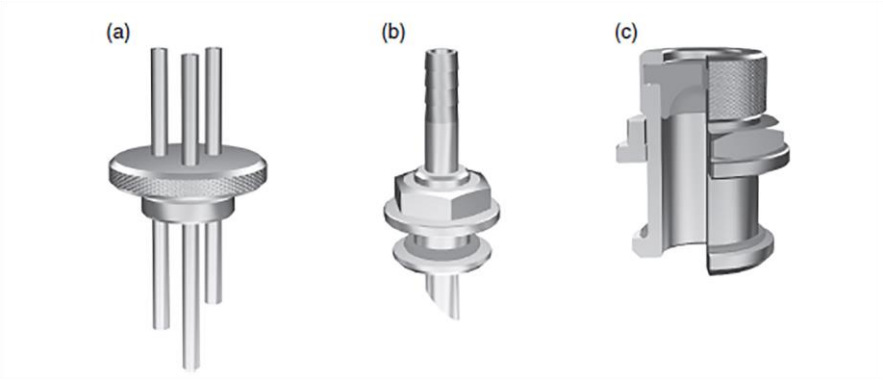

Figure 2.14. Methods of introducing fluids. (a) Triple inlet; (b) single inlet; (c) septa (Matthews, 2008).

Spargers are devices through which sterile air is introduced in the fermentation vessel.
They are located at the bottom of the fermentation tank and glass wool filters are used for sterilization of air and other gases. The sparger pipes contain small holes of about 5-10 mm, through which pressurized air is released in the fermentation medium. In Figure 2.15a the conventional 'L' design, for high gas flow rates, typically up to 2 vessel volumes per minute, is illustrated.
The alternative reported in Figure 2.15b consists of a 'microporous' design, used for gas flow rates typically up to 0.1 vessel volumes per minute. Gas is pushed through a fine sintered tip.
Each pore is approximately 15 mm and so gas enters the liquid phase in a very fine dispersion of high surface area, thus improving mass transfer.

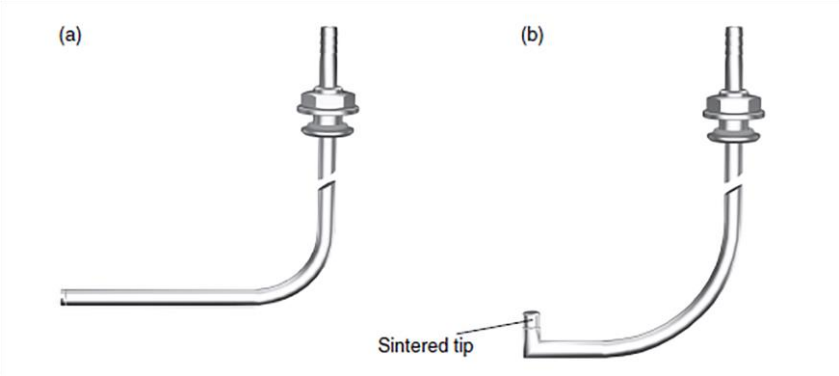

Figure 2.15. Example of spargers. (a) L sparger; (b) sintered sparger (Matthews, 2008).

Sampling system: During the fermentation process, sampling is required to monitor the fermentation process and to perform a quality control. Although every time a sample is picked up the system is exposed to contamination, this phase is unavoidable to check cell count, cell viability, medium constituents, and compounds produced that cannot be read online. The system should be steam sterilized and completely aseptic before withdrawal of sample.

A sampling device for a small-scale reactor is shown in Figure 2.16.

Figure 2.16. Sampling system for an autoclavable bioreactor (Matthews, 2008).

Probes for pH, temperature and DO_2 control. In particular, pH is controlled by a probe, which is part of a feedback loop that adds acid or base, allowing the vessel content to remain at the optimum set point. The temperature is also controlled on a feedback loop with information coming from a temperature probe in the vessel. The maintenance can be realized by using water circulating jackets or coils placed around the vessel: a local controller activates the power supply, or the opening and closing of solenoid valves for hot/cold water supply. Another important parameter to be considered during a fermentation process is the dissolved oxygen, except for anaerobic processes.

Oxygen is not very soluble in culture media (only 7 ppm at 37 °C) but can be rapidly consumed in large amounts by microorganisms. For this reason, the knowledge of the DO_2 level in the vessel and its control at the required set point are fundamental.

Various other probes can be used, if sufficient head plate ports are available and the placing of probes does not alter too drastically the mixing profile of the medium.
These could include optical density (OD) probes, that can be used as indicators of biomass, or devices for monitoring the dissolved CO_2, that could inhibit the cell growth.
In Figure 2.17, a typical configuration of a bioreactor equipped with sparger, baffles and an impeller with three Rushton turbines is shown. The common geometrical ratios between the design parameters are reported.

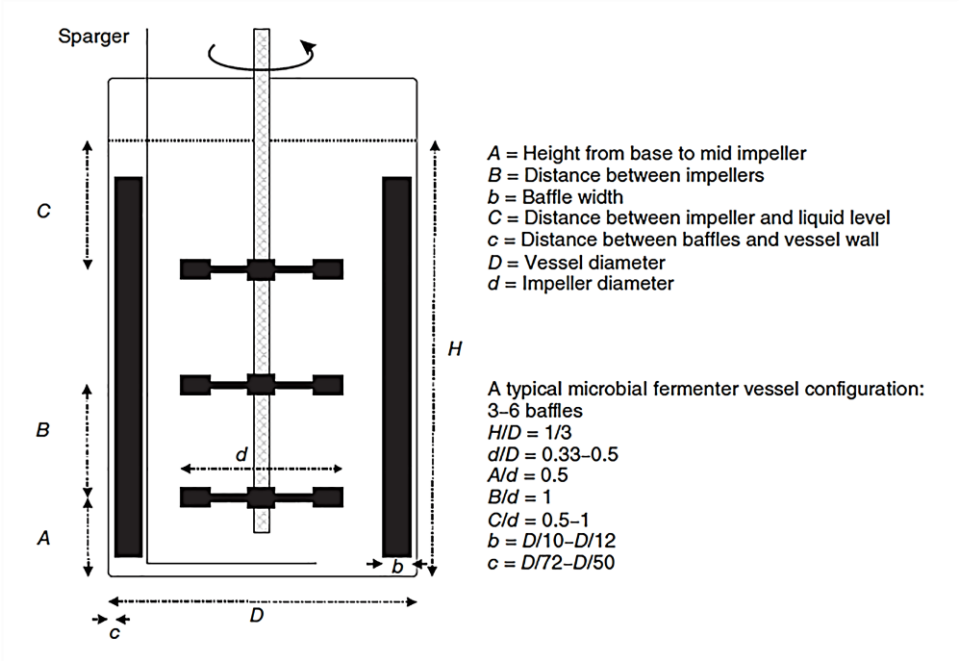

Figure 2.17. Standard design parameters for a microbial bioreactor vessel with typical values included (Meyer et al., 2017).

2.1.4 Fermentation technologies for probiotic and postbiotic production

The process design and the fermentation technologies adopted for the production of functional pro- and postbiotic products influence all the main bioengineering variables, i.e., yield, productivity and final product concentration (Paulová et al., 2013). Maintaining a yield close to its maximal theoretical value and obtaining a high product concentration decrease the costs of raw materials and downstream processes, respectively. Moreover, an increase in productivity and a reduction in non-productive periods can contribute to efficiently use the manufacturing equipment.

Industrial processes for probiotic production mainly use conventional batch fermentation with suspended cells, due to its simplicity and low risk of contamination, but the maximum potential of the process is often not achieved. Substrate and product inhibition, presence of dead times, and low productivity are the main problems of this process arrangement (Paulová et al., 2020).

In a fed-batch process, substrate inhibition is avoided and a subsequent shortening of the lag phase occurs, while a correctly designed feeding of nutrients can increase the product concentration and the process productivity (Paulová et al., 2020).

The highest productivity of the process is guaranteed when the fermentation is carried out by a continuous configuration, despite the high volumes at low concentration of the desired product coming from the system, together with the increased risk of contamination, make the continuous methodology quite problematic.

With regard to probiotics, preservation of the microorganism viability during fermentation, downstream processes, storage, and digestion in the host's upper gastrointestinal tract represents a key parameter for developing probiotic foods. Several approaches have been investigated to enhance cell viability, based on the application of sublethal stresses during fermentation to improve cell resistance to some environmental changes and to enhance their long-term stability.

For these purposes, addition of protectants, cell protection by microencapsulation (Lacroix & Yildirim, 2007; Ross et al., 2005), increasing sublethal temperatures, salt or bile salt treatments (Doleyres & Lacroix, 2005) could be applied.

Although very few literature data have been reported on continuous fermentations with probiotics until now, continuous cultures can lead to both high cell yield and process volumetric productivity, as well as decreasing the demand for downstream processing capacity (Doleyres et al., 2002). Moreover, continuous fermentation technology allows to produce cells with different physiologies and to apply stresses under well-controlled conditions, by easily manipulating some environmental parameters, such as the medium composition and the dilution rate.

Another recently investigated technology consists of a membrane system with continuous feeding of fresh medium: cells are retained in the bioreactor by ultrafiltration or microfiltration membrane whereas small molecules diffuse through the pores of the membrane according to their size. Therefore, inhibitory metabolic products are eliminated in the permeate and cells are concentrated on the retentate side. The concentrated cell fraction can be harvested batch-wise or continuously with minimal additional downstream treatment for cell concentration before freezing or freeze-drying.

In membrane bioreactors, cells are subjected to several stresses, such as low nutrient concentration, oxygen, osmotic and mechanical stresses that could affect sensitive bacteria, but might also lead to cross-protection effects for other stresses (Lacroix a$ Yildirim, 2007).

In recent years, probiotic fermentation with immobilized cell technology has been investigated.

Cell immobilization has been used to perform high cell density fermentations for both cell and metabolite production. The main advantages are the improved resistance to contamination and bacteriophage attack, enhancement of plasmid stability, prevention from washing-out during continuous cultures, and the physical and chemical protection of cells towards the environmental stresses (Lacroix et al., 2005). In particular, it seems that the cells produced by immobilized cell technology might exhibit physiology profiles that are better suited for adaptation to growth in the very competitive conditions of the gastrointestinal tract, but this remains to be demonstrated (Lacroix & Yildirim, 2007).

2.2 Drying techniques

Dehydration, or drying, is one of the unit operations most commonly used for food preservation. Drying consists of a moisture removal process with a simultaneous heat and mass transfer:

1. transfer of thermal energy from the surrounding environment to the evaporation surface, or transfer of electromagnetic energy into the material;
2. transfer of internal moisture to the surface of the food material and its subsequent evaporation to the surroundings.

The main purpose of drying is to ensure food stability during storage. Reduction in water activity inhibits microbial growth and enzyme activity, but the processing temperature is usually insufficient to cause their inactivation: any increase in moisture content during storage, for example due to faulty packaging, will result in rapid spoilage. The reduction in weight and bulk of food reduces transport and storage costs. Moreover, for some types of food, dehydration provides a convenient product for the consumer or more easily handled ingredients for food processors.

Drying time, temperature, and water activity influence the final product quality.

Low temperatures generally have a positive influence on the quality but require longer processing times. Low water activity (lower than 0.8) retards or eliminates the growth of microorganisms and enzymatic reactions, but results in higher lipid oxidation rates, as shown in Figure 2.18.

In fact, the resistance to oxidation becomes not relevant at water activities around 0.3.

Maillard browning reactions peak at intermediate water activities (0.6 to 0.7), indicating the need for a rapid transition of the medium to low water activities and the subsequent importance of process times.

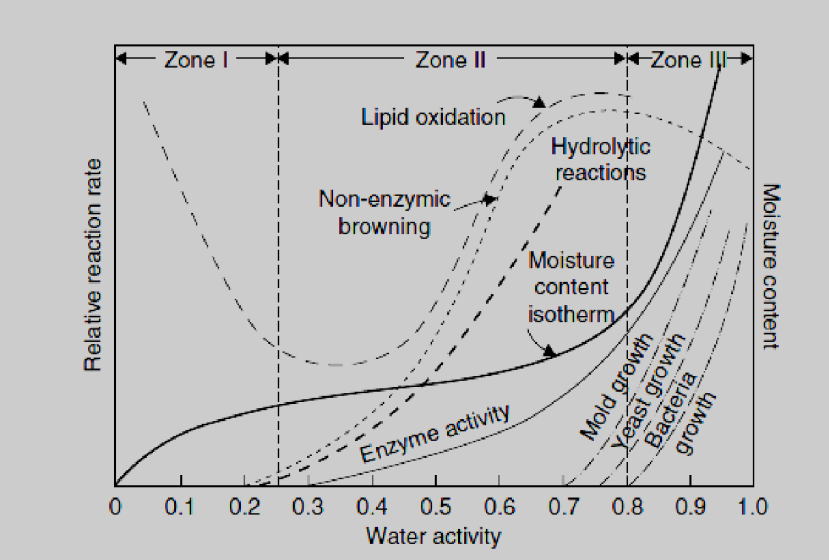

Figure 2.18. Relationship between food water activity and moisture content and the relative rates for several chemical reactions, enzyme activities and microorganism growths that lead to food deterioration (Krochta, 2007).

Drying process and storage can reduce the quality of food products, in comparison with fresh material, in terms of texture, loss of flavour or aroma, changes in colour and nutritional value.

For these reasons, the aim of improved drying technologies is to minimise these changes, maximising the process efficiency.

The main drying methodologies used are:

- Application of heated air (convective or direct drying), such as tray or cabinet driers, tunnel dryers or spray dryers. Air heating reduces air relative humidity, which is the driving force for drying. Higher temperatures speed up diffusion of water inside the solids, so drying is faster. However, product quality considerations limit this application: too hot air could almost completely dehydrate the solid surface, leading to shrinkage or collapse of internal pores and to crust formation. Moreover, too high temperatures could denature bioactive compounds of interest in the final product;
- Indirect or contact drying (heating through a hot wall), as drum drying, vacuum drying;
- Dielectric drying (radiofrequency or microwaves absorbed inside the material). It is the focus of intense research nowadays. It may be used to assist air drying or vacuum drying;

- Freeze drying is increasingly applied to dry foods, for pharmaceutical or medical applications. It keeps biological properties of proteins, and retains vitamins and bioactive compounds;
- Supercritical drying (superheated steam drying) involves steam drying of products containing water. The water in the product is boiled off, and joined with the drying medium, increasing its flow. It is usually employed in closed circuits and allows a proportion of latent heat to be recovered by recompression, a feature which is not possible with conventional air drying.
  It may have a great potential for foods if carried out at reduced pressure, in order to lower the boiling point;
- Natural air drying takes place when materials are dried with unheated forced air, taking advantage of its natural drying potential. The process is slow and weather-dependent, so a wise strategy "fan off-fan on" must be devised considering the following conditions: temperature, relative humidity and moisture content of the air, and temperature of the material being dried.
  The total time (including fan off and on periods) may last from one week to various months, if a winter rest can be tolerated in cold areas.

2.2.1 Freeze Drying

The heat used to dry foods removes water and therefore preserves the food by a reduction in water activity. On the other hand, the heat could cause a loss of sensory characteristics and nutritional qualities. In freeze drying, a similar preservative effect is achieved by reduction in water activity without heating the food. Absence of air during processing, together with low temperature, prevents deterioration due to oxidation or modifications of the product, and allows nutritional qualities and sensory characteristics to be better retained. In particular, volatile aroma compounds are trapped in the food matrix, the texture of freeze-dried foods is well maintained, and their open porous structure allows a rapid and full rehydration, but it is fragile and requires protection from mechanical damage. Moreover, there are not important changes to proteins, starches or other carbohydrates.
However, freeze-drying operation is slower than conventional dehydration technologies.
Energy costs for refrigeration are high and the production of a high vacuum is an additional expense. This, together with a relatively high capital investment, results in high production costs for freeze-dried foods. For these reasons, freeze drying process is essentially used to dry expensive foods which have more delicate aromas or textures (for example, coffee, mushrooms, herbs and spices, fruit juices, meat, seafoods, vegetables and complete meals for military rations or expeditions).
Microbial cultures for use in food processing are also freeze dried for long-term storage prior to inoculum generation. In Table 2.2, the main differences between freeze drying and conventional hot air drying are shown.

Table 2.2. Main differences between conventional drying and freeze drying (Fellows, 2000).

Conventional drying	***Freeze drying***
Successful for easily dried food (vegetables and grains)	Successful for most foods but limited to those difficult to dry by other methods
Meat generally unsatisfactory	Successful with cooked and raw meats
Temperature range 37-93°C	Temperatures below freezing point
Atmospheric pressure	Reduced pressures (27-133 Pa)
Evaporation of water from surface of food	Sublimation of water from ice front
Movement of solutes and sometimes case hardening	Minimal solute movement
Stresses in solid foods cause structural damage and shrinkage	Minimal structural changes or shrinkage
Slow and incomplete rehydration	Rapid and complete rehydration
Solid or porous dried particles often having a higher density than the original food	Porous dried particles having a lower density than other food
Odour and flavour frequently abnormal	Odour and flavour usually normal
Colour frequently darker	Colour usually normal
Reduced nutritional value	Nutrients largely retained
Costs generally low	Costs generally high, up to four times those of conventional drying

Freeze drying mainly uses the sublimation phenomenon (at temperatures lower than 0.01 °C and vapor pressures below 0.612 kPa) to eliminate a certain amount of the water from a product.

Three important steps characterize the freeze-drying process: freezing, sublimation (or primary drying stage) and desorption (or secondary drying stage). As shown in Figure 2.19, if a product at the pressure and temperature corresponding to ambient conditions (point A) has to be freeze-dried, it will follow the path from point A to point D: the product should first be frozen by decreasing its temperature, then the water vapor pressure should be lowered below the pressure corresponding to the triple point and finally some heat should be supplied to help the ice to convert into vapor by sublimation. After all the ice has been sublimated (and during sublimation), desorption of non-freezable water occurs.

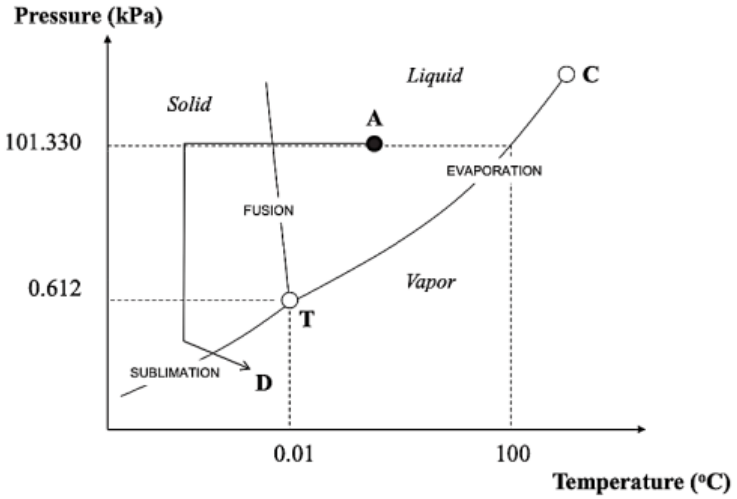

Figure 2.19. Water phase diagram.

Figure 2.20 shows a schematic diagram of a food product during freeze-drying at different stages of the process. The frozen food is placed in a vacuum chamber on a shelf plate which supplies the necessary energy for sublimation and desorption by conduction (q_C). The product can also receive heat from the top shelf and the surroundings by radiation (q_R). Convection is rare because very few fluid molecules are available under vacuum so the purely convective heat transfer coefficient should be negligible in high vacuum situations. Thus, heat in the freeze-drying chamber is mainly transferred to the product by radiation and/or conduction from the shelf plates. As the product receives heat, sublimation is initiated (t_1). Drying is faster during primary drying due the availability of large amounts of unbound water in the frozen state. Ice sublimation leaves a porous dry layer that increases as drying proceeds (receding front). During sublimation, two distinct phases separated by a receding front are present in the product: the dry layers and a frozen core.

During secondary drying (t_2), bound water has to be lost: a major portion of the bound water is in the unfrozen state and the drying rate is very slow.

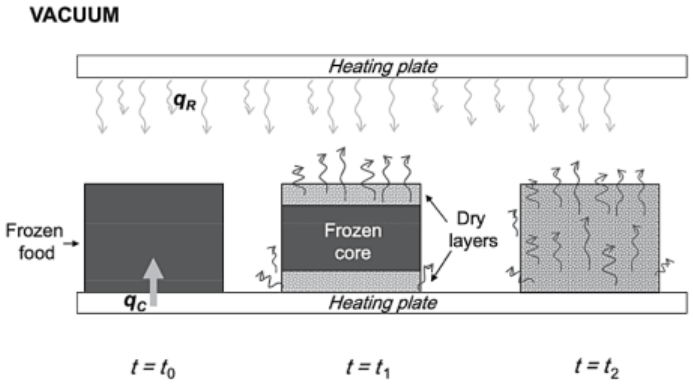

Figure 2.20. Schematic representation of a food product during freeze-drying at initial time (t_0), during sublimation (t_1) and when desorption (t_2) takes place. q_C: conduction heating; q_R: radiation.

Freeze-drying is widely used to stabilize probiotic microorganisms, owing to a very minimum heat stress exerted on the living cells. The freezing is the key process step that determines the drying behaviour of the sample: in order to ensure a rapid vapour migration during drying, large and contiguous ice crystals in the suspension should be present. On the other hand, these large ice crystals can induce cell damage due to mechanical stress, providing structural alteration of foods and affecting the microbial viability in probiotics. For these reasons, optimum freezing conditions often require a compromise between the requirements of the bacteria and the drying performance.

Therefore, high cell viability in probiotic freeze-dried products depends on the process conditions but also by the cryoprotectant effect of polysaccharides (Zayed & Roos, 2004) and proteins (Burgain et al., 2015) present in the food matrices to be processed.

2.2.2 Spray Drying

Spray drying is one of the most important techniques to produce dried powders.

The principle of working is based on moisture removal by application of heat to the feed product, controlling the humidity of the drying medium. Here, the uniqueness is that the evaporation of moisture is promoted by spraying the feed into a heated atmosphere, resulting in an improved drying rate. This resulting rapid evaporation maintains a low droplet temperature so that high drying air temperatures can be applied without affecting the product. Furthermore, the time of drying is very short in comparison with most other drying processes. Therefore, low product-temperature and short drying- times allow very heat-sensitive products to be spray-dried.

For these reasons, spray drying technique is used to dry pharmaceutical fine chemicals, foods, dairy products, blood plasma, numerous organic and inorganic chemicals, rubber latex, ceramic powders, detergents, and other products (Figure 2.21).

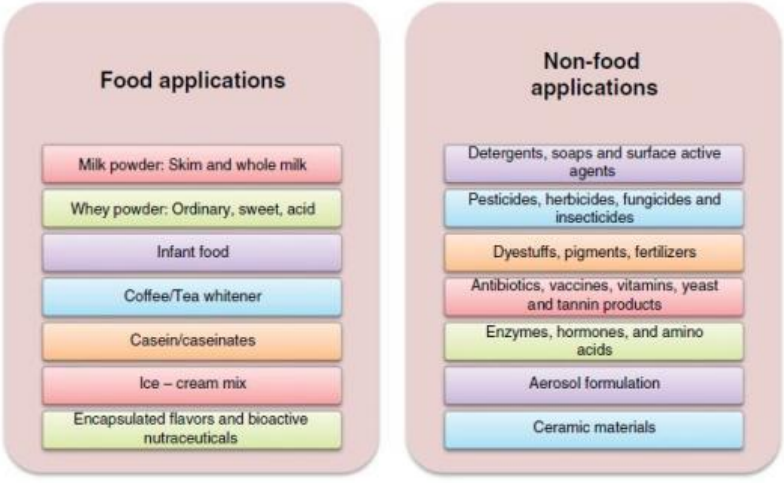

Figure 2.21. Applications of spray drying technique.

In Figure 2.22 the main steps of a typical spray drying process, consisting of atomization, air-droplet contact, moisture removal and product separation, are schematized.

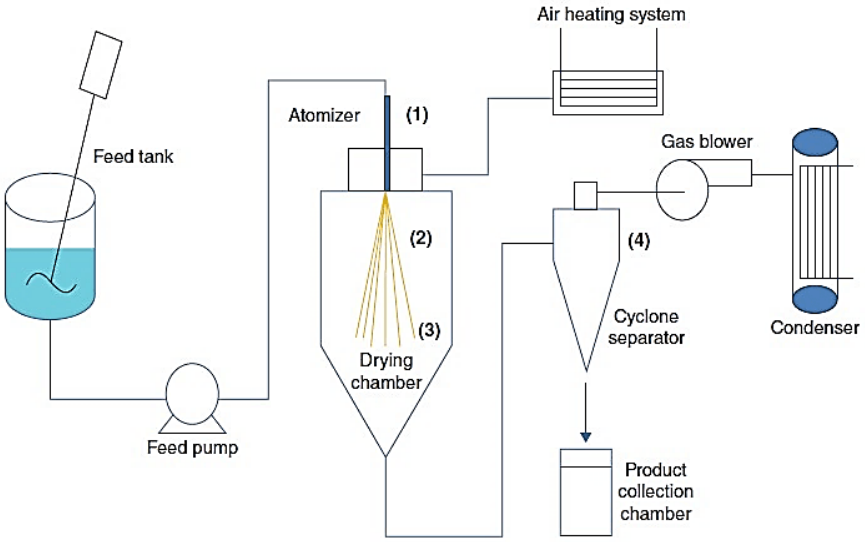

Figure 2.22. Main steps of the spray drying process. (1) Atomization; (2) Spray-hot air contact; (3) Evaporation; (4) Separation (Anandharamakrishnan et al., 2015).

1) **Atomization** of feed solution, consisting of the breakup of the liquid bulk into a large number of droplets, reducing the internal resistances to moisture transfer from the droplet to the surrounding medium. An enormous increase in surface area of the bulk is observed as the droplet fission proceeds: this greater surface-to-volume ratio enables spray drying to achieve faster drying rates (as drying time is proportional to the square of the particle dimension).
   In addition, atomization is a fundamental stage owing to its influence on shape, structure, velocity and size distribution of the droplets, and, in turn, particle size and nature of the final product.
   A cubic meter of liquid forms approximately 2×10^{12} uniform 100 micron-sized droplets, with a total surface area of over 60000 m^2 (Masters, 2002). Consequently, there will be a minimal loss of heat sensitive compounds and particles with the desired morphology and physical characteristics will be obtained.

Atomizers can be classified in:

- *Rotary atomizers* (Figure 2.23): a feed liquid is centrifugally accelerated at high velocity to the centre of a rotating wheel with a peripheral velocity of 200 m/s.

  In rotary atomizers more uniformly sized droplets (30 ÷ 120 μm.) are produced; since the necessary atomization energy is supplied by the rotating wheel, the feed supply unit can operate at low pressure than that required in hydraulic and pneumatic nozzle atomizers. Rotary atomizers present difficulties in handling viscous feed.

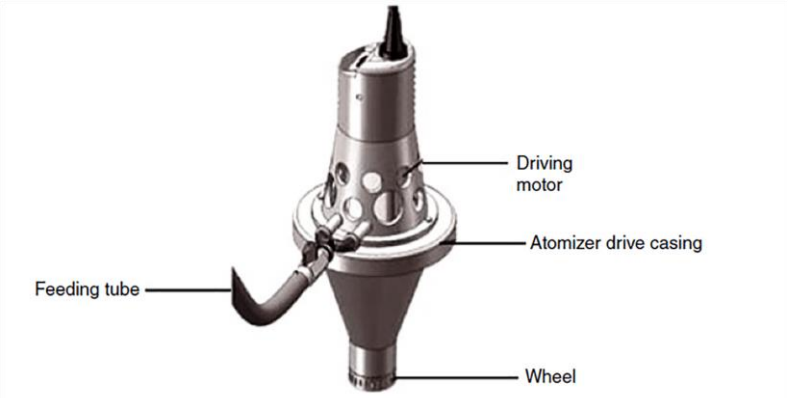

Figure 2.23. Rotary atomizer (Anandharamakrishnan et al., 2015).

- *Pressure nozzle (or hydraulic) atomizers* (Figure 2.24a): a liquid under pressure is discharged through an orifice: pressure energy is converted to kinetic energy, and feed emerging from the nozzle orifice as a high-speed film readily breaks into a spray of droplets (mean droplet size 120 ÷250 μm). At high feed rates, sprays are generally less homogeneous and coarser than rotary atomizers.

- *Two-fluid nozzle atomizers* (Figure 2.24b): the break-up of a liquid is based on the impact with high-velocity air or other gaseous flow. Compressed air creates a shear field, which atomizes the liquid and produces droplets with mean size in the range 30 ÷ 150 μm.

  Twin-fluid nozzles are capable of handling highly viscous feed. These atomizers produce much finer and more homogeneous spray when compared to pressure nozzles.

  A further disadvantage of this type of liquid-gas nozzle is the “downstream turbulence” caused by large gas flows used

(a) **(b)**

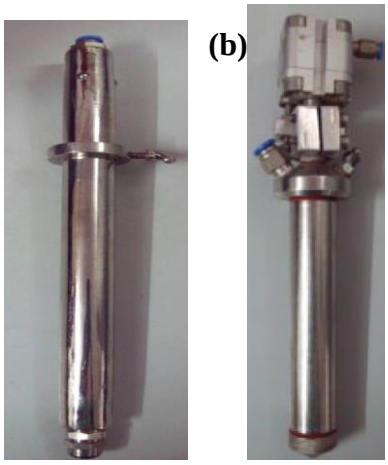

Figure 2.24. Pressure nozzle (a) and twin-fluid pressure nozzle (b) atomizers (Anandharamakrishnan et al., 2015).

- *Ultrasonic atomizers* (Figure 2.25): the fluid to be atomized initially passes over the surface of vibrating piezoelectric disks, which sets ultrasonic vibrations within the liquid.
  These vibrations cause molecules on the surface of the liquid to move, lowering their surface tension. Then the fluid passes through an amplifier and reaches a resonant surface.
  An increase in the amplitude of vibration causes the droplet formation: uniform droplet size distributions are obtained, with shorter residence times. Ultrasonic nozzles allow a higher retention of the active components present in feed and have been effectively used for the drying of probiotic cells (Semyonov et al., 2011), in order to obtain higher viability.

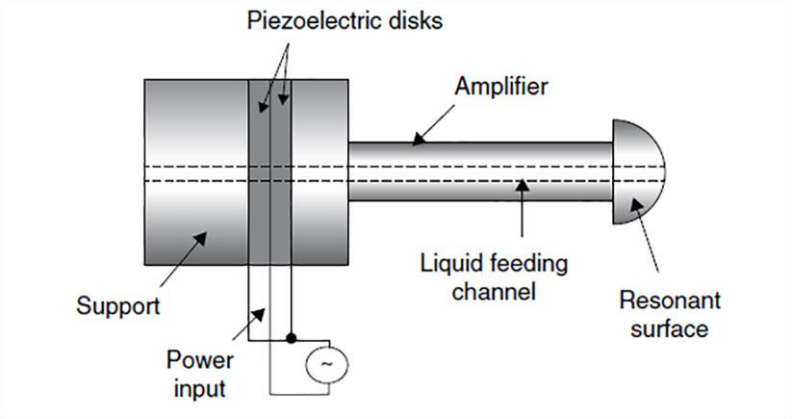

Figure 2.25. Ultrasonic atomizer (Anandharamakrishnan et al., 2015).

- *Electrohydrodynamic atomizers* represent a recent technique for atomizing a feed liquid by using electrospray or electrohydrodynamic sprays.

In the electrospray, electrical potential is applied to the needle to introduce free charge at the liquid surface. The high intensity of electric current applied between two oppositely electrodes of an electrospray system allows the production of droplets of narrow particle size distribution.

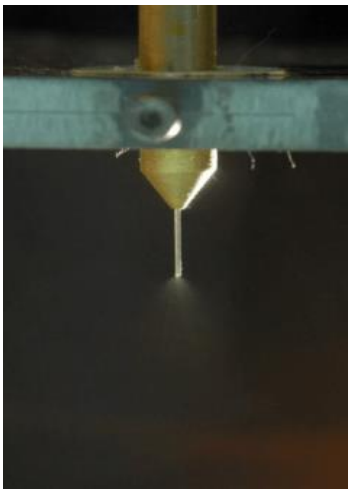

Figure 2.26. A spray emerging from an electrohydrodynamic atomizer (Anandharamakrishnan et al., 2015).

2) **Contact of spray with hot air**: during spray-air contact, the droplets usually meet hot air in the spraying chamber, either in co-current flow or counter-current flow (Figure 2.27).
In *co-current configuration*, the product and the drying medium pass through the dryer in the same direction. In this arrangement, the atomized droplets entering the dryer are in contact with the hot inlet air, but their temperature is kept low due to the high evaporation rate which takes place and is approximately at the wet-bulb temperature.
The contact time of the hot air with the spray droplets is only a few seconds, during which drying is achieved, and the air temperature drops instantaneously. As the droplet movement is co-current with the air flow, the driving force for heat and mass transport reduces significantly after the initial droplet–air contact. Low temperatures and low residence times allow to avoid the thermal degradation of heat sensitive products.
In *counter-current and mixed flow configurations*, the atomized droplets are injected into the chamber in the opposite direction of the inlet hot air flow. For this reason, the system works with a sustained increase in the heat and mass transfer driving force along the dryer; the particles are exposed for a longer time in relatively higher temperature and this might not be suitable for heat sensitive materials.

In the mixed flow arrangement, both co-current and counter-current air-droplet flows are realized along the system and, also in this case, the drawback is the higher exit temperature of the product.

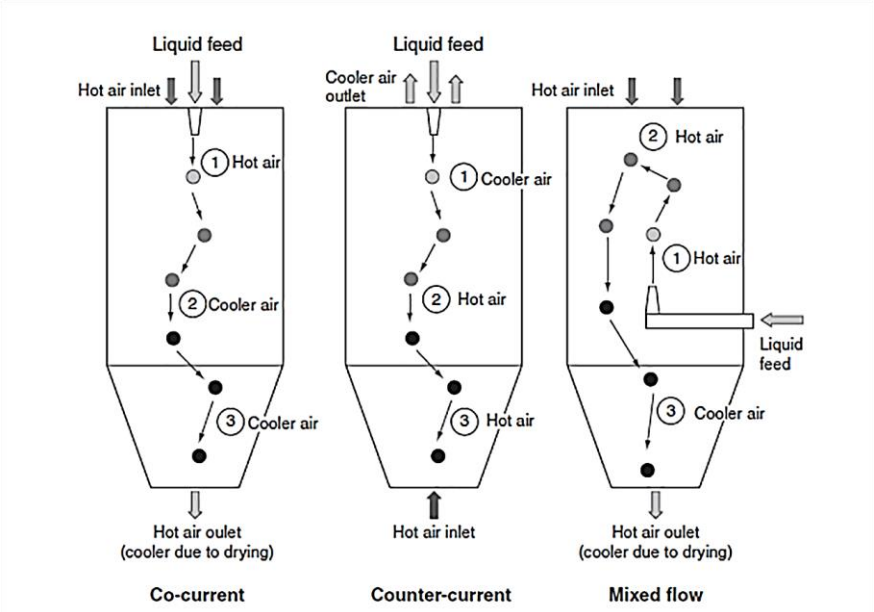

Figure 2.27. Typical configurations used in the spray drying process (Woo et al., 2013).

3) **Evaporation of moisture**: Evaporation of moisture during spray drying can be visualized as two stages: (i) constant rate period; and (ii) one or more falling rate periods.

When the droplets are exposed to hot gas, rapid evaporation takes place. During this constant rate period, free moisture is removed constantly from the surface of the droplet, where the temperature is constant at the wet-bulb value.

Wet bulb temperature is that the drying gas reaches when it is saturated with vapor from the liquid. As the moisture removal from the droplet proceeds, the particle dimension reduces, as shown in Figure 2.28 (step 1); the solute dissolved in the liquid reaches a concentration beyond its saturation concentration and a thin shell at the droplet surface tends to form (crust formation). This stage represents the falling rate period and consists of a diffusion-controlled phase where the evaporation rate depends on the water vapor diffusion through the dried shell of the product (Figure 2.28, step 2-3).

An interesting phenomenon that happens during the falling rate period is the “bubble formation”, as shown in step 4 of Figure 2.28. When the partial pressure of moisture vapor at the droplet centre exceeds ambient pressure, it could result in bubble formation and a subsequent increase in temperature.

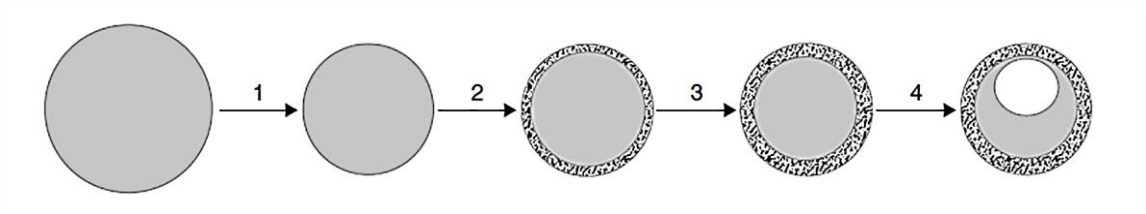

Figure 2.28. A representation of a droplet history during spray drying process (Anandharamakrishnan et al., 2015).

4) **Particle separation**. The primary and secondary stages of particle separation involve withdrawal of dried product from the conical bottom of the spray chamber and its removal using dry collectors or wet scrubbers. The spray drying chamber often has a conical bottom to facilitate the collection of the dried powder, performed using screw conveyors or pneumatic systems. However, the product separation stage is complete when also the powder entrained in the drying gas stream is recovered. For this purpose, a cyclone separator, a bag filter or electrostatic precipitator can be used, depending on the size of the particles carried away by the exhaust gas and on the final product specifications.

   A cyclone separator utilizes centrifugal force to separate the solid particles from a carrier gas. The gas stream enters tangentially, forming an outer vortex as it travels downward.

   The centrifugal force imparted by the increasing air velocity causes particle separation, while an inner vortex is created in the opposite direction, allowing the exit of clean gas from the top.

   In a bag filter, the drying gas loaded with particles enters the hopper at the centre or bottom, and travels through the filter, where the particles are retained on the surface of the bag.

   Instead, electrostatic precipitation is a method of particle collection that uses electrostatic forces. It consists of imparting a charge on the individual particles in the gas stream and driving the charged droplets towards the oppositely charged collection electrode.

   The particles are then removed from the surface of the collector electrode, usually by a rubber spatula or by rappers, which vibrate the collection surface to facilitate their recovery.

Chapter 3

3 Materials and methods

3.1 Strain and feedstock

The microorganism used for the fermentation process is *Lactobacillus paracasei* (LP) CBA L74, patented and provided by Kraft-Heinz Company. It is a homofermentative, facultative anaerobic and mesophilic bacterium with an optimal growing temperature of 37°C; its selective culture medium is DeMan, Rogosa & Sharpe (MRS) broth. The Heinz's strain was isolated from infant's faeces and was selected based on its capability to grow in milk and cereals, rice in particular, and on its safety that was tested according to the "Guidelines for the evaluation of probiotic in Food" (2002) of the Joint FAO/WHO working group. In addition, a genetic characterization of the strain was done, necessary for a commercial probiotic strain. It is conserved at -26 °C in cryovials with glycerol (20%), reactivated through incubation (37°C, 24 h) in animal free broth (AFB, Appendix A1), in order to prepare the inoculum of the fermentation. AFB was considered as a suitable alternative to the optimal MRS broth for lactobacilli growth and was chosen owing to the absence of ingredients of animal nature. Two different revitalization protocols for lab and pilot scales were used (Appendix A2).

For lab scale trials, a commercial liquid skimmed milk (Granarolo®) was used, while rice flour and dried navy beans (Ontario crop) were provided by Heinz Italia S.p.A.

At pilot scale trials, powder skimmed milk and rice flour were provided by Kraft-Heinz Italia S.p.A.

For both laboratory and pilot scale trials, dextrose and amylase were provided by Sigma-Aldrich (Germany) and by Kerry (Canada), respectively.

In Table 3.1 and Table 3.2, the recipes used for rice flour, milk and beans at laboratory scale, and for rice flour and milk at pilot scale, respectively, are reported.

Table 3.1. Recipes used for fermentation trials on rice flour, milk and bean at laboratory scale.

	Ingredient	***% (w/v)***
Lab-scale recipe for rice	Rice flour	15
	Water	83.988
	Dextrose	1
	Amylase	0.012
Lab-scale recipe for milk	Skimmed milk (wet)	98
	Dextrose	2
Lab-scale recipe for beans	Dried beans	10
	Water	90

Table 3.2. Recipes used for fermentation trials on rice flour and milk at pilot scale.

	Ingredient	***% (w/v)***
Pilot-scale recipe for rice	Rice flour	15
	Water	83.988
	Dextrose	1
	Amylase	0.012
Pilot-scale recipe for milk	Skimmed milk (powder)	10.5
	Water	87.5
	Dextrose	2

3.2 Experimental equipment

3.2.1 Laboratory scale apparatus

Pre-treatment

Enzymatic pretreatment of rice flour by amylase was carried out into the same bioreactor used for the fermentation stage, described below.

To obtain the bean-based fermenting substrate, dried beans were soaked, cooked and blended in a thermostatic mixer (Hot mix PRO TWIN-cutter) equipped with two vessels: a vessel with an anchor impeller used for soaking and cooking, and a vessel with sharp blade impeller for blending.

Figure 3.1. Thermostatic mixer used to pre-treat dried beans before fermentation.

Fermentation

The apparatus used at laboratory scale to perform the fermentation process consists of a non-continuous system using a batch reactor. This type of system is more versatile and allows a greater control of the process conditions, as well as the sterilization of the reactor and the substrates to be fermented. The entire system, shown in Figure 3.2, is mainly composed of:

- Vessel
- Mixing system
- Thermal conditioning system
- Peristaltic pump for fueling sodium hydroxide, needed for pH control.

Figure 3.2. Laboratory scale fermentation apparatus.

The vessel is a cylindrical Pyrex tank (10 cm internal diameter) with a total volume of 1.5 L (Figure 3.3a). It is equipped with an external jacket for the circulation of a service fluid (deionized water) coming from a thermostatically controlled water bath, that allows to control the temperature inside the reactor during the entire fermentation process. The thermo-stated bath (Haake G), shown in Figure 3.2b, is connected through silicon tubes to the external jacket of the vessel.

It is supplied with a resistance to heat the fluid and a cooling coil to cool it and the temperature parameters can be manually set by the operator. The mixing system consists of a stainless-steel rotating shaft mounted on the head plate and equipped with two or three Rushton turbines with different dimensions (Figure 3.3c and Figure 3.3d).

A largest turbine is mounted at the bottom of the shaft, while one or two smaller turbines of equal size are located in the upper area, in order to avoid the contact with the pH probe inserted inside the reactor during the mixing (Figure 3.4).

The entire mixing system is connected to a motor that allows the adjustments of the stirring speed.
A silicone gasket placed on the edge of the vessel and a metal ring around the head plate ensure hermetic sealing of the reactor and consequently an appropriate isolation of the reactor interior from the external environment. The head plate has four ports (Figure 3.3e) that allow the addition of probes; the ports not used can be hermetically sealed with stainless steel plugs.
In this case, the head plate has 2 working inputs, one for inserting a InPro 3100I/150 probe (Mettler Toledo, Milan, Italy) connected to a transmitter M300 (Mettler Toledo, Milan, Italy), useful for in line temperature/ pH measurements, while the second one is connected through a small silicon tube to a tank containing the NaOH buffer (0.2 M), fed by a peristaltic pump for pH control (Figure 3.3f). The peristaltic pump is a Watson Marlow 400Q series 401 u/D1; the tube used has an external and internal diameter of 4 and 2 mm, respectively; the speed is of 15 rpm and the flow rate is of 3-5 mL/min.

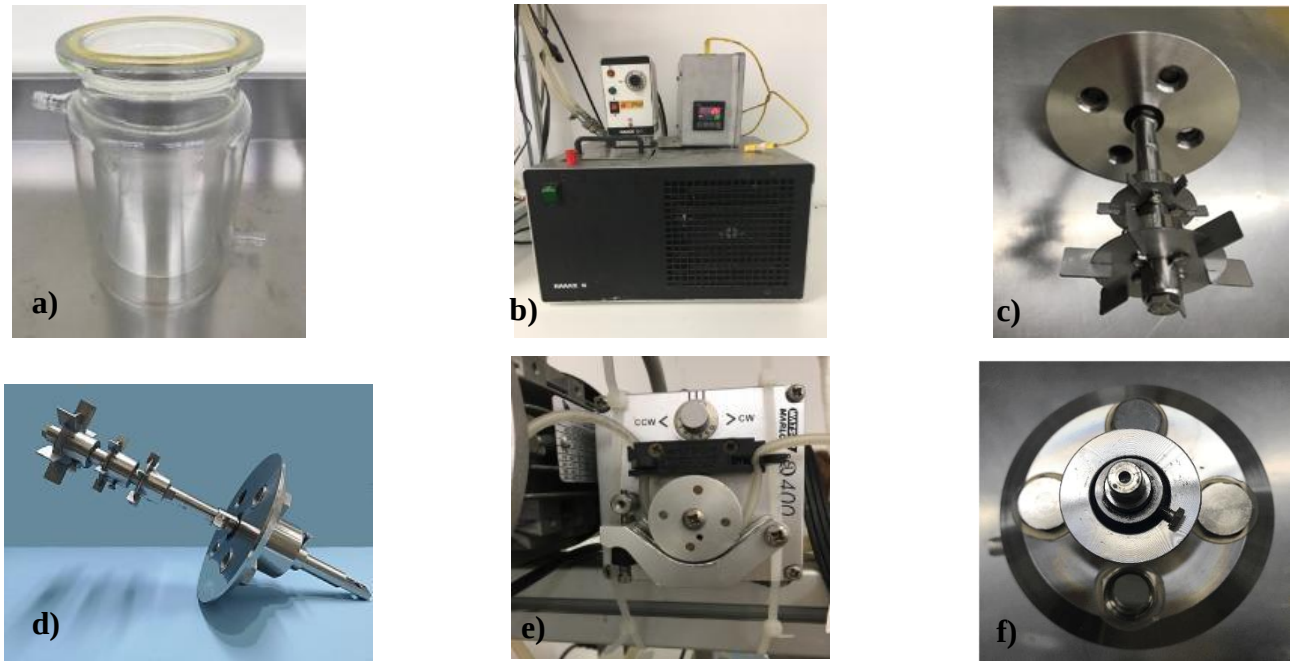

Figure 3.3. Main parts of the laboratory scale fermentation system: a) reactor, b) thermal conditioning system c) impeller with two Rushton turbines (I1); d) impeller with three Rushton turbines (I2); e) head plate; f) peristaltic pump.

Figure 3.4. Rushton turbines with different dimensions.

The impeller with two Rushton turbines (I1) was used for agitation of rice and milk substrates, while a three Rushton system (I2) was designed to improve mixing during bean fermentation.
Figurative schemes of the bioreactor constituted by the vessel and the mixing systems I1 and I2, with the relative characteristic dimensions, are shown in Figure 3.5. All the parameters and aspect ratio described in the paragraph 2.1.3 for the design of a bioreactor were respected.

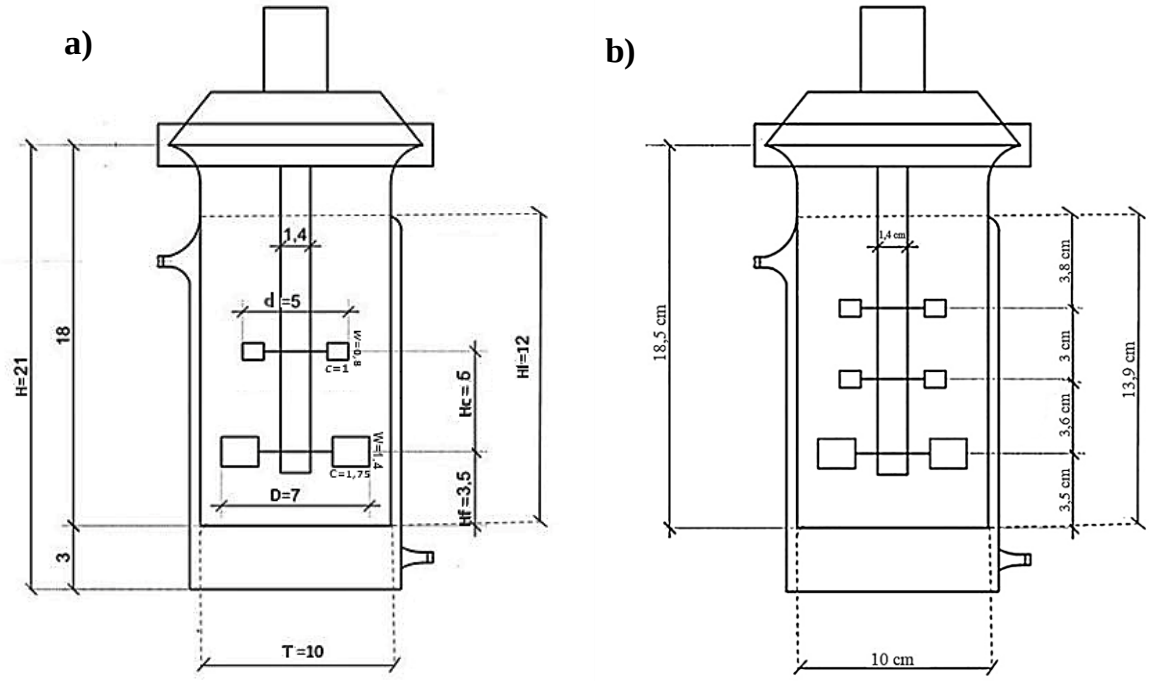

Figure 3.5. Figurative schemes of lab scale bioreactors with their respective characteristic dimensions: a) bioreactor designed for milk and rice fermentation (I1); b) bioreactor designed for bean fermentation (I2).

Drying apparatus

At lab scale two different drying technologies were used to dry the product in order to understand the different impact that the freeze drying and the spray drying methods have on it.

The **freeze-drying process** was carried out in a benchtop freeze dryer (Alpha 1-2 LD plus, Christ), with height of 345 mm, a width of 315 mm, a depth of 460 mm, and a weight of approximatively 28 kg. It is shown in Figure 3.6. It consists of four components:

- a vacuum drying chamber with three internal shelves and 8 rubber valves for drying in round bottom flasks;
- an ice condenser chamber with an internal ice condenser for binding the water vapour released by the product;
- an external vacuum pump for generating vacuum inside the drying chamber (Varian Rotary vane DS 102, pumping speed of 5 m^3/h and an ultimate total pressure with gas ballast of 2 × 10^{-2} mbar);
- aeration and media drain valve.

Figure 3.6. Bench Freeze Dryer.

Spray drying of the fermented samples produced at lab scale was performed in collaboration with Tecoma Drying Technology S.r.l., located in Maranello (MO)-Italy, using a lab scale spray dryer, shown in Figure 3.7, with the following specifications:

- Dimensions: 750 × 650 × 1200 mm^3;
- Weight: 110 kg;
- Atomizer material: stainless steel;
- Nozzle type: 2 fluid nozzles;
- Air flow: 0 ÷ 330 m^3/h;
- Evaporation Capacity: 1.5 L/h;
- Inlet air temperature: 30 ÷ 250° C ± 1°C;
- Outlet air temperature: 30 ÷ 120 °C ± 1°C;
- Particle size range: 1-25 μm;
- Maximum sample feed: 2000 mL/h;

- Minimum sample volume: 30 mL;
- Power: 3500 W;
- Heater power: 3000 W.

Figure 3.7. Laboratory scale spray dryer.

3.2.2 Pilot scale apparatus

Fermentation and drying apparatus

The pilot plant, shown in Figure 3.8, is currently installed in 57- Heinz Innovation Centre in Nijmegen, The Netherlands.

It consists of a continuous system, so each phase can be performed in line.

The plant mainly consists of the following unit operation stages, schematized in Figure 3.9:

- Powder slurry preparation (mixing tank);
- I Heating;
- Buffering (Buffer tank);
- II Heating;
- Cooling;
- Fermentation (batch bioreactor);
- Spray drying.

The mixing tank has a maximum volume of 500 L and it is directly connected with line water supply (all other ingredients to be fermented are dosed outside and then added manually).

The product is sent from the mixing tank to the buffer tank through a screw pump, with a flow rate of 200 L/h, passing through a tube system in which the first direct steam injection takes place.

The plant is provided with a saturated steam at 4 bar: the quantity of steam supplied to the system is controlled by a PID (proportional–integral–derivative) feedback control.

In particular, the amount of steam needed to heat up the product is regulated by the opening of a steam valve, based on the temperature that the product has to reach, that is read by a probe.
The buffer tank has a maximum capacity of 300 L, an external jacket supplied with water to control the temperature of the product, and it is also equipped with a mixing system to guarantee the homogeneity of the product. The buffer tank is connected with the fermenter through tubes where the second direct steam injection and the cooling phase take place. The II steam injection works with the same mechanism explained above. Cooling takes place in a tube heat exchanger in which the exterior tube is fed with water at 20°C. The product is sent to the bioreactor with a flow rate of 250 L/h.
The bioreactor has a maximum capacity of 300 L and it has an external jacket in which a thermo-stated fluid allows the control of the temperature inside; also, in this case the system is a batch culture type. The mixing system, shown in Figure 3.10, is equipped with an impeller with a customized inclined blade turbine and is connected to a motor to control the mixing speed.
The fermenter has 4 ports, connected to:

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

The fermenter has a valve in the lower part, connected to a low-pressure steam system, that allows sterile sampling throughout the fermentation process.
The spray dryer is connected to the fermenter through an external pump. It consists of a vertical axis spray dryer, GEA's PRODUCTION MINORTM model (Figure 3.11a), with a co-current flow and two fluid nozzle atomization. At the bottom of the cyclone, the product is collected in a bucket, shown in Figure 3.11b. The spray dryer has the following specifications:

- dimensions: $4.4 \times 2 \times 2.7$ m^3;
- weight: 1300 kg;
- atomizer material: stainless steel
- nozzle type: two liquid nozzle;
- air flow: max 360 kg/h;
- water evaporation capacity: max 35 kg/h;
- inlet air temperature: max 350°C;
- outlet air temperature: max 110°C;
- particle size range: $10 \div 90$ µm;

- feeding pump flow rate: peristaltic pump with variable remote control.

Figure 3.8. Photo of the pilot plant located in the 57-Kraft Heinz Innovation Center.

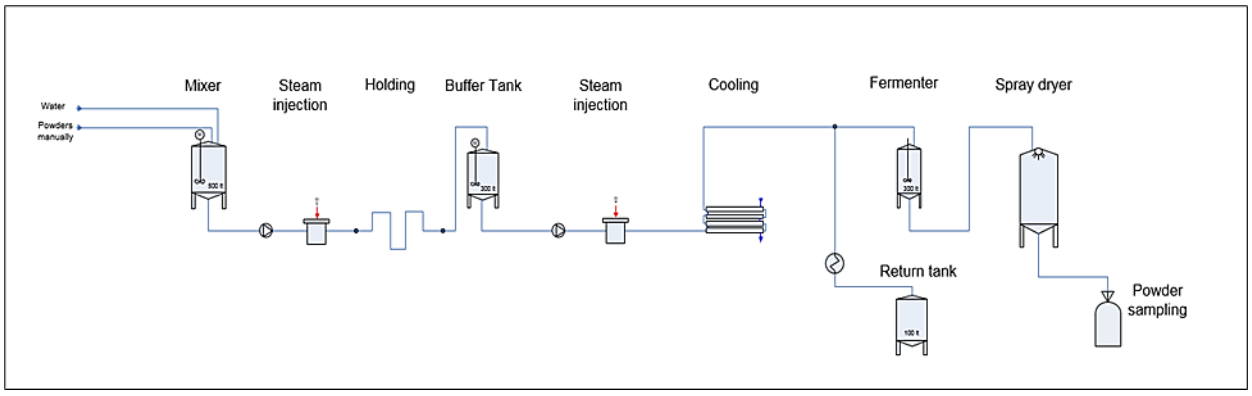

Figure 3.9. Figurative scheme of the pilot plant.

Figure 3.10. Mixing system of the pilot bioreactor.

a)  **b)** 

Figure 3.11. Drying and separation systems used at pilot scale: a) spray drier; b) cyclone and collection bucket.

3.3 Laboratory scale experimental procedures

In the following paragraphs, the main procedures used at laboratory scale for obtaining wet and dried semi-finished products will be described. Pretreatments of substrate, fermentation, inactivation, and drying were optimized and appropriate protocols were adopted for each substrate.

A preliminary set of operations was performed for all three substrates:

- sterilization of each component of the apparatus (vessel, mixing system, and pH probe);
- revitalization of the strain (Appendix A2) and preparation of the reactor;
- After pre-treatment of the matrix, the entire reactor was assembled, loaded under sterile conditions, and then connected to the motor and to the thermostatic bath. Only for rice, the pre-treatment phase was carried out directly inside the assembled reactor, as described below.

3.3.1 Laboratory scale protocols for rice flour

Rice flour pre-treatment

An enzymatic hydrolysis of rice flour using amylase was performed to prevent starch gelatinization during the heat treatment needed for the feedstock sterilization and to guarantee an efficient mixing and homogeneity of the suspension during the fermentation stage. Moreover, amylase treatment allowed to process a higher amount of flour than that it would be possible to ferment in absence of the enzymatic hydrolysis step, without affecting the sterilization and mixing performances.

As reported in the work of Gallo et al., 2021, where fermentation tests were carried out on hydrolysed wheat flour suspension, a higher amount of solid can be processed, that results in improved fermentation performances and a higher final postbiotic productivity.

The pre-treatment consisted of the following steps:

- adding water and amylase into the reactor and bringing the system at 65°C (for the enzyme activation);
- adding rice flour and dextrose;
- buffering at 65°C for 10 min;
- bringing the system at 85°C and holding for 20 min (for the inactivation of the enzyme);
- sterilization of the pre-treated suspension in autoclave with a standard cycle (121°C for 20 min).

Fermentation and microbial inactivation

- The bioreactor (equipped with the mixing system I1, described in the paragraph 3.2.1) was heated at a temperature of 37 °C, setting the thermostatic bath connected to the outer jacket of the vessel at this temperature. When needed, pH of the feedstock was adjusted at 6.2 before starting the fermentation, using 0.2 M NaOH buffer, previously prepared.

- After adding the inoculum, fermentation was carried out for 24 h, without and with pH control (fermentation protocols RF1and RF2, respectively), with a stirring speed of 60 rpm. During the process, samples were withdrawn aseptically under a sterile hood at specific times (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) and for each sample, bacterial growth, pH, metabolite production and sugar consumption were measured. In order to avoid contamination of the medium and an excessive reduction of the fermenting volume, it was necessary to carry out at least 6 fermentation tests to have a triplicate of each data with a minimum number of collected samples, for each fermentation test.

- Microbial inactivation of the fermented product was carried out by autoclaving the reactor at 90°C, with a holding phase of 1 min. In literature, efficient heating treatments to kill this microorganism were carried out at 85°C for 20 s or 95 ÷ 100°C for 15 s (Cuomo et al., 2013; Sarno et al., 2014). However, technological limits have led us to choose the above operative T/t conditions (90 °C/1 min) to reach an efficient microbiological deactivation (< 10^2 CFU/mL).

Freezing and Freeze Drying

Freezing of the fermented samples, performed separately from the drying apparatus.

Petri plates (90 mm diameter) were filled with 10/15 mL of fermented sample and stored at -26°C for 24 h, before the freeze-drying process. After a warm up of the vacuum pump for 15 min, frozen samples were placed on the freeze dryer shelves (the maximum capacity for the shelves inside the drying chamber is 9 Petri dishes); freeze drying process lasted 24 h and consisted of a main drying phase at a temperature of -20°C and a pressure of 1.0 mbar for 12 h, and a final drying phase for the removal of the residual moisture at a temperature of -56.5 °C and a pressure of 0.017 mbar for 12 h.

At the end of the process, the vacuum pump was switched off and the drying chamber was aerated via the aeration valve; then the product was removed from the unit. Defrosting of the air condenser was carried out at room temperature.

Spray drying

Drying of the fermented products produced at lab scale was performed in collaboration with Tecoma Drying Technology S.r.l. located in Maranello (MO), Italy, using a laboratory scale spray dryer described in the paragraph 3.2.1. The spray-drying process was divided in 4 phases:

- pre-filling of the supplied liquid;
- nebulization of the liquid in small droplets through an atomizer;
- contact between the droplets and the controlled temperature air flow;
- separation and collection of the solid product.

The operative parameters were: feed flow of 20 mL /min, air flow of 200 m^3/h, air inlet temperature of 180 °C and an air outlet temperature of 80 °C.

In these conditions, a sample of 500 mL was dried in less than 30 min.

Fermented samples, dried by freeze and spray drying, were rehydrated reproducing the same 1:15 w/v ratio of the fermenting medium; then the residual bacterial charge, lactic acid and functional metabolite content were measured to check the effect of these drying techniques on the properties of the final rice-based postbiotics.

3.3.2 Laboratory scale protocols for milk

Preliminary phases

Sterilization of dextrose dissolved in a little amount of water (30 mL) in autoclave with a standard cycle (121°C for 20 min) and preparation of 500 mL of 0.2 M NaOH buffer were performed.

The commercial milk used for the laboratory scale processes did not need to be sterilized, so it was directly loaded into the reactor with the sterilized dextrose solution, under sterile hood.

Fermentation and microbial inactivation

The fermentation process on milk was carried out using the same procedure and the same operative conditions described for rice in paragraph 3.3.1. The process was performed without and with pH control (fermentation protocols: MF1 and MF2, respectively) using the mixing system I1 described in the paragraph 3.2.1. Microbial inactivation of fermented milk was performed, also in this case, by autoclaving at 90 °C for 1 min.

Drying processes

In order to dry the fermented milk product, the same freeze drying and spray drying procedures adopted for rice samples were used for milk (paragraph 3.3.1).

Fermented milk powders were rehydrated reproducing the same 1:10 w/v ratio of the fermenting medium; then the residual bacterial charge, the lactic acid and the functional metabolite content were measured.

3.3.3 Laboratory scale protocols for beans

Bean pre-treatment

Preliminary studies were performed to find an appropriate way to pre-treat the raw material (dried white beans) in order to obtain an adequate fermenting medium in terms of sterilization and homogeneous mixing during the process.

For this purpose, feasibility tests were performed milling the whole beans with a commercial bladed grinder and loading the reactor with 1000 mL of the resulting flour suspension (10% w/v). The starch gelification was allowed by a thermal treatment at 85°C for 1 h in agitation, followed by a conventional autoclave cycle at 121°C for 20 min.

The first results showed great difficulties in terms of adequate mixing during the process due to high viscosity of the fermenting suspension and presence of contaminants in the medium.

In order to find optimal strategies to remedy these problems, the granulometric dimension of the bean flour obtained was fixed ($d_{average}$ = 110 µm), having experimentally noticed a correlation between different particle sizes, different starch gelation conditions, and a consequent different medium viscosity. That was confirmed also by Luhovyy et al. (2017), which reported the effect of the particle size on the physicochemical properties of navy bean flours, such as total starch content, starch damage, water absorbance capacity and viscosity conditions.

Subsequently, several enzymatic pre-treatment and sterilization conditions were proposed:

- BT1: Thermal treatment at 85°C for 1 h in agitation to allow starch gelification, followed by an autoclave cycle at 121°C for 20 or 40 min into reactor or in Pyrex glass bottles ($d_{bottle} < d_{reactor}$);

- BT2: Enzymatic treatment with α-amylase (0.012% w/v) at 85°C for 1 h in agitation, followed by an autoclave cycle at 121°C for 20 min into the reactor.
- BT3: Enzymatic treatment with α-amylase (0.024% w/v) at 85°C for 1 h in agitation, followed by an autoclave cycle at 121°C for 20 min or at 134 °C for 40 min into the reactor.
- BT4: Dry thermal treatment of flour in oven at 121°C for 1.5 h; enzymatic treatment with α-amylase (0.024% w/v) at 85°C for 1 h in agitation; then an autoclave cycle at 134°C for 40 min into the reactor.
- BT5: Dry thermal treatment of flour in oven at 121°C for 1.5 h; enzymatic treatment with α-amylase (0.024% w/v) at 50°C for 30 min then at 85°C for 1 h in agitation; final autoclave cycle at 134 °C for 40 min into the reactor.

Unsatisfactory results in terms of adequate mixing and sterilization led to the realization of a completely different way to pre-treat beans:

- BT6: Dried navy beans were soaked in deionized water (1:10 w/v) for 24 h at room temperature. Cooking of the soaked beans was performed in a thermostatic mixer at 95°C for 2 h in agitation. Subsequently, the beans were blended inside the same mixer at 95°C for 40 min and the resulting suspension was loaded into the reactor and sterilized in autoclave at 134 °C for 40 min.
  The cooking phase allowed a preventive starch gelification and a first sterilization of the medium; then the strong autoclave cycle guaranteed a complete sterilization and absence of contamination. In Figure 3.12, soaked beans before cooking and blending are reported.
  Soaking and cooking water was not discharged in order to test the ability of LP CBA L74 to metabolize Raffinose-Series Oligosaccharides (RSO) sugars (raffinose and stachyose, in this case) during the fermentation process.

Figure 3.12. Beans after soaking.

Fermentation and microbial inactivation.

The system was cooled down to 37°C and the strain was inoculated to start the fermentation process. Fermentation was carried out for 24 h, without pH control and with a mixing stirring of 81.4 rpm. Two different mixing conditions were tested, using both impellers (I1 and I2) described in the paragraph 3.2.1. As well as for milk and rice, the process was monitored by sampling at specific times in order to evaluate pH, bacterial growth, metabolite production and sugar consumption, as the fermentation proceeded.

The heat treatment for microbial inactivation was performed at different conditions:

- BI1: Reactor in autoclave at 90°C per 1 min.
- BI2: Reactor in autoclave at 90°C for 3 min.
- BI3: Reactor in autoclave at 90°C for 5 min.
- BI4: Reactor in autoclave at 90°C for 30 min.
- BI5: Pyrex glass bottles (ID 6.3 cm) filled with fermented bean suspension in autoclave at 90°C for 20 min.

Drying processes

In order to dry the fermented bean product, the same freeze drying and spray drying procedures adopted for rice and milk samples were used for beans (paragraph 3.3.1).

Fermented bean powders, obtained by freeze and spray drying, were rehydrated, reproducing the same 1:10 w/v ratio of the fermenting medium; then the residual bacterial charge, lactic acid and functional metabolite content were measured. In Table 3.3, the different fermentation protocols used at lab scale for each substrate, rice flour, milk and beans, are summarized.

Table 3.3. Fermentation protocols performed on rice, milk and beans at lab scale.

Substrate	Fermentation Protocols	Description
Rice	RF1	Without pH control
	RF2	With pH control
Milk	MF1	Without pH control
	MF2	With pH control
Bean	BF_1	Mixing system I1
	BF_2	Mixing system I2

3.5 Physical properties of fermented rice, milk and beans

To investigate the effect of fermentation process on some physical properties which influence the application of dried products as functional ingredients in food formulations, freeze dried fermented rice flour, milk and beans obtained at laboratory scale were characterized in terms of:

- *water absorption capacity* (WAC) and *water adsorption index* (WAI), which represent the water binding capacity of a product and are influenced by its protein and carbohydrate content. Low WAC is desirable for making thinner gruels, while high WAC values are desirable for the formulation of baked products, which require high hydration to improve the handling properties of doughs;
- *oil holding capacity* (OHC), which measures the ability of food materials to absorb oil and retain aromas and flavours;
- *foaming capacity* (FC), which is dependent on the presence of soluble protein molecules which decrease the surface tension of water. It corresponds to the percent increase in volume of the solution owing to foam formation.

For WAC and WAI determination, 1 g of sample was mixed in a vortex with 10 mL of distilled water or olive oil for 1 min and then centrifuged at 5000 g for 30 min.

After separation, the volume of supernatant was recorded and used for determination of water absorption, according to the Equations (17) and (18). The same procedure was used for OHC, except that olive oil was used instead of water, using Equation (19).

$$\text{WAC} = \frac{V_{initial\ water} - V_{supernatant}}{m_{weighed\ solid}} \quad (17)$$

$$\text{WAI} = \frac{m_{deposit\ after\ centrifugation}}{m_{weighed\ solid}} \quad (18)$$

$$\text{OHC} = \frac{V_{initial\ oil} - V_{supernatant}}{m_{dry\ solid}} \quad (19)$$

For FC calculation, 2 g of samples were weighed and added to 50 mL of distilled water, inside a 100 mL graduated cylinder. The suspension was mixed and shaken and the total volume after 30 s was recorded. The percentage increase in volume after whipping is calculated using the following Equation (20).

$$\text{FC} = \frac{V_{after\ whipping} - V_{before\ whipping}}{V_{after\ whipping}}\ x\ 100 \quad (20)$$

3.6 Pilot scale experimental procedures

Pilot scale trials were performed only on rice flour and milk. Previous studies carried out on rice and milk substrates allowed for a rapid and effective optimization of the lab-scale protocols so that pilot scale experiments could be carried out. Conversely, fermentation of a leguminous substrate had been an unexplored field until then; for this reason, preliminary feasibility tests and more detailed investigations at laboratory scale were needed, to search for the best conditions to carry out the fermentation process on beans, including the determination of the most appropriate pre-treatments before fermenting and the best reactor conditions.

Since the pilot plant is a continuous system, each phase of the protocol was performed in line.

Most of the procedures were common for milk and rice, except for the substrate pre-treatment phase.

As well as for lab scale, a preliminary phase was necessary for the sterilization and preparation of the equipment (some steps were done in the laboratory, others in the pilot plant):

Laboratory

- Pre-inoculum preparation (10 mL) and incubation for 24 h at 30°C (Appendix A2);
- inoculum preparation (2 L) and incubation for 24 h at 30°C (Appendix A2);
- inoculum valve and glassware sterilization in autoclave;
- preparation of 4 L of NaOH 30% w/v (7.5 M). A more concentrated NaOH was used at pilot scale, necessary to buffer larger volumes (200 L) than those of the lab scale (1 L).

Pilot Plant

- Weighing of all the ingredients to be used to ferment;
- draining and filling of the mechanical seal;
- calibration of the probe for pH/ temperature measurements;
- checking of the air filter;
- cleaning of the line (with cleaning solutions);
- sterilization of the UHT line;
- deaeration of the fermenter;
- connection and steam flushing of the inoculum and sodium hydroxide valves;
- sterilization of the fermenter using steam at 121°C for 30 min.

3.6.1 Pilot scale protocols for rice flour

Pre-treatment, fermentation and inactivation phase

All ingredients (rice flour, water, dextrose, amylase) were added and mixed for 20 min in the mixing tank. A sampling was performed to check the dry matter.

First direct steam injection was carried out at 65°C to obtain activation of amylase.
The product was held inside the buffer tank for 20 min to ensure the optimal activity of the enzyme, then a second direct steam injection at 137°C was conducted to inactivate the enzyme and, at the same time, sterilize the product. After a cooling phase, the fermenter was filled with the medium to be fermented and the system was brought to 37°C. Inoculum was added and a sampling was carried out to check the dry matter. Fermentation was carried out for 24 h (samples are taken every 2 h; night shifts were necessary); then the fermented rice was inactivated inside the same bioreactor, bringing the system at 90°C and holding for 1 min (the temperature of the fluid in the outer jacket of the bioreactor was set at 90°C).

Spray drying

Spray drying method was used to dry the fermented rice suspension.
Immediately after the fermentation and the inactivation phases, the fermented product was sent to the spray dryer using a peristaltic pump. The feeding pump flow rate was automatically regulated on the basis of the outlet temperature set.
The process parameters were:

- Inlet air temperature: 210°C;
- outlet air temperature: 95°C;
- Air flow-rate: 450 m^3/h.

The inlet and outlet air temperatures could be adjusted in order to have a specific moisture content in the final product. Moisture content in rice was < 3.5% at the end of the process and the throughput of the spray dryer was 0.45 kg $_{powder}$/h.

3.6.2 Pilot scale protocols for milk

Fermentation and inactivation phase

Procedures to process milk at pilot scale were the same used for rice flour fermentation and inactivation, except for the absence of the enzymatic treatment, carried out for rice flour inside the buffering section. Powder milk, water, and dextrose were added and mixed for 20 min in the mixing tank. A sampling was performed to check the dry matter.
First direct steam injection was carried out at 85-90°C while the second steam injection at 137 °C.
After cooling the product, the fermenter was filled, heated at 37 °C, and inoculated (a check of dry matter was performed also in this case). Milk fermentation lasted 24 h, and samples were collected every 2 h, as well as for rice.

Spray drying

The same spray drying protocol used for rice was adopted for milk. Moisture content in milk was < 5% at the end of the process and the output flow of dried product was 0.45 kg $_{powder}$/h.

3.7 Analytical methods

Each fermentation test was characterized in terms of microorganism's growth, presence of contaminants, organic acids production (lactic, butyric, propionic, and acetic acids), pH, sugar consumption and a functional bioactive peptide:

- Bacterial growth was monitored by serial dilutions and spread plate method on Petri plates (Appendix A4) filled with MRS agar (Oxoid, Basingstoke, UK; Appendix A5).
  Gelatine Peptone Bios Agar (Biolife; Milan, Italy) (Appendix A6), MacConkey agar (Oxoid, Basingstoke, UK) (Appendix A7), and Mannitol Egg Yolk Polymyxin (MYP) Agar (Oxoid, Basingstoke, UK; Appendix A8) were used to control the presence of microbial contaminants (coliform microorganisms, aerobic microorganisms and *Bacillus Cereus*, respectively*)* in the fermenting medium. All plates were incubated at 37 °C for 48 h before reading.
  The bacterial charge measured was expressed in CFU/mL, where CFU are the colony forming units. Special anaerobic kits (Anaerogen Compact, Oxoid) were used for MRS plates to guarantee growth anaerobic conditions for LP CBA L74 during the incubation period.
  In order to determine the biomass concentration expressed in g/L, the cell weight was estimated as described below. The inoculum broth, with a bacterial load of 10^8 CFU/mL previously measured by a spread plate method, was centrifuged and the supernatant was discharged.
  The pellet was weighed and a specific biomass weight of 10^{-12} g/CFU was calculated.

- pH was measured automatically using a probe linked to the peristaltic pump and controlled in a range of 5.8 ÷ 6.2 using sodium hydroxide (0.2 M).

- The concentration of lactic acid was determined by High Performance Liquid Chromatography (HPLC), Agilent Technologies 1100, equipped with an Agilent Zorbax C18 column (150 mm × 4.6 mm and a pore size of 8 μm) with a Visible/UV detector.
  The eluent was 1% $NH_4H_2PO_4$ aqueous solution at pH of 2.7 modified with H_2PO_4, with a flow rate of 0.8 mL/min. The detector was set at 218 nm.

- Secondary metabolites, as acetic, propionic, and butyric acids, were measured by gas chromatography, (GC), Agilent technologies 6890, using a capillary Poraplot Q column (25 mm × 0.32 mm). The mobile phase was helium gas with a flow rate of 200 mL/min.

These secondary acids could be produced by competitive bacteria and their detection in the fermented substrates usually indicates contamination. Before the acid analysis, the samples were pretreated to guarantee protein precipitation (Appendix A9).

- Fructose, glucose, sucrose and lactose concentrations were determined by HPLC, Agilent Technologies 1100, with RI detector, using a Rezex RHM-Monosaccharide column (300 mm × 7.8 mm and a pore size of 8 μm). The eluent was 3.5 mM H_2SO_4 aqueous solution with a flow rate of 0.6 mL/min. Raffinose and stachyose concentrations were determined by HPLC, Agilent technologies 1100, with a Visible/UV detector, using an Agilent Zorbax Eclipse Plus C18 column (100 mm × 4.6 mm and a pore size of 3.5 μm). The eluent was 1.7 mM H_2SO_4 aqueous solution with a flow rate of 0.5 mL/min. The detection was set at 191.5 nm.
- Starch content was determined using a Total Starch Assay Kit (AA/AMG), Megazyme, through spectrophotometric analysis at 510 nm.
- The bioactive peptide reputed as the functional metabolite responsible of the immunomodulatory activity of the obtained postbiotics was detected and quantified by HPLC-DAD, Agilent 1100 binary pump, with Visible/UV detector, using a Phenomenex Synergi™ Fusion-RP C18 column (250 mm × 4.6 mm and a pore size of 80 Å). The detection wavelength was set at 215 nm.
  A buffer A (0.05% TFA + 2% CH_3CN) and a buffer B (0.05% TFA + 90% CH_3CN) were used as eluents, by a gradient consisting of 10-30% B in 22 min and a flow rate of 1 mL/min.

3.8 Kinetic study

The mathematical modelling of fermentation processes allows the formulation of predictions about the kinetics of biomass growth and metabolite production.

The kinetic knowledge of these processes plays a key role in designing and developing improved strategies for optimizing, controlling and scaling up biological process systems, to ensure their economical convenience. Unsegregated and unstructured kinetic models were proposed to describe the fermentation processes performed at lab scale on rice, milk and beans in terms of bacterial growth, lactic acid and functional metabolite production.

3.8.1 Microorganism's growth

The time, t_d (h), needed for the microorganism to double in number during the growth exponential phase was calculated using Equation (21):

$$t_d = \frac{t_{exp}}{n} \quad (21)$$

where t_{exp} is the duration of the exponential phase and n is the number of generations, calculated by Equation (22):

$$N_f = N_0 * 2^n \quad (22)$$

N_i and N_f are the numbers of cells at the beginning and at the end of the growth exponential phase, respectively. The constant growth rate, k (h^{-1}), is defined in the Equation (23):

$$k = \frac{n}{t_{exp}} = \frac{1}{t_d} \quad (23)$$

The growth rate r_x can be expressed by a first order kinetic respect to the biomass concentration, according to Equation (2).

Applying the separation of variables and the integration with the initial condition ($X=X_0$ at $t = t_0$), it is possible to obtain the following Equation (24):

$$\mu = \frac{ln\left(\frac{X}{X_0}\right)}{t - t_0} \quad (24)$$

where t_0 (h) is the initial time of the growth exponential phase and X_0 (g/L) is the corresponding biomass concentration.

Multiple-substrate growth models were proposed to express the specific growth rate μ as a function of each substrate concentration by an additive model (Beyenal et al., 2003), showed in Equation (25):

$$\mu = \frac{\mu_1 (S1) + \mu_2 (S2) + \cdots + \mu_i (Si)}{i} \quad (25)$$

Each μ_i (S_i) was correlated to the corresponding substrate concentration S_i by the Monod model, which describes μ as a function of substrate concentration, the Haldane model, which takes into account a carbon substrate inhibition on the biomass growth and the Monod model corrected with an additional term to consider a possible product inhibition, according to Equations (26), (27) and (28):

$$\mu_i (S_i) = \mu_{max} \frac{S_i}{S_i + K_{S_i}} \quad (26)$$

$$\mu_i (S_i) = \mu_{max} \frac{S_i}{S_i + K_{S_i} + (S_i^2 / K_{I_i})} \quad (27)$$

$$\mu_i (S_i) = \mu_{max} \frac{S_i}{S_i + K_{S_i}} \frac{k_p}{k_p + P} \quad (28)$$

where μ_{max}, S_i, K_{si}, K_{Ii}, k_p are the maximum specific growth rate (h^{-1}), the substrate concentration (g/L), the semi-saturation constant (g/L), the substrate inhibition constant (g/L), and the product inhibition constant (g/L), respectively.

3.8.2 Lactic acid and functional metabolite production rate

For all three substrates, lactic acid production rate, r_p, and functional peptide production rate were modelled by the Luedeking−Piret model, reported in Equation (14).

3.9 Shelf-life analysis

Shelf-Life analysis consists of a collection of analytical, chemical, microbiological and sensory data over time in order to study how a product changes as it ages and understand the point at which a food product becomes unacceptable or harmful for the consumer. It represents an indispensable evaluation for food industries in order to define the storage, the distribution conditions and the limits.

Shelf-life analysis was performed on dried fermented milk and rice produced at pilot scale.

The analysis evaluated:

- Organoleptic characteristics (taste, texture, appearance);
- Microbiological characteristics (microbial load);
- Chemical characteristic (lactic acid concentration).

The products were stored in sealed vacuum pouches at three different temperatures, 4 °C, 20 °C, and 37°C (accelerated shelf-life), in order to understand how the storage temperatures affect the considered parameters.

These shelf-life tests started at the end of July 2020 and were carried out for six months.

Sensory analysis was performed at “Heinz Innovation Centre” in Nijmegen -The Netherlands.

The organoleptic characteristics were evaluated according to the following protocol, performed by trained panellists (at least 10 panellists for each test; no precise information about the number of panellists was provided).

Tasting protocol:

1) Weighing of 15 g of product;
2) Visual evaluation of powder;
3) Addition of 150 mL of hot water to have dissolution;
4) Tasting and off-flavours evaluation;
5) Filling sensory evaluation form.

Microbiological and chemical analysis were performed following the analytical methods explained in the paragraph 3.5 for the determination of the bacterial load and organic acid concentration (spread plate method and lactic acid analysis by using HPLC-UV), respectively.

Before performing these analyses, the powder was resuspended, reproducing the same proportions of the fermentation batch (15% and 10% of dry matter for rice and milk, respectively), comparing these results with the wet fermented samples.

3.10 Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016®.

Fermentation tests, lactic acid, starch, sugar and functional metabolite analysis were carried out in triplicate and for each experimental data, mean values and standard deviations (n = 3) were calculated. Their statistical significance was evaluated by Student's t-test, accepting as significant only results with $p < 0.05$. Microsoft Excel 2016® Solver® was used to estimate the kinetic constants of each model, solving non-linear regressions by non-linear Generalized Reduced Gradient (GRG) method. The solver provides the values for the kinetic parameters that minimize the objective function, the Residual Sum of the Squares (RSS), calculated as the sum of squared differences between experimental and theoretical data obtained for the specific growth rate, as in Equation (29).

$$RSS = \sum_{i} (\mu_{experimental} - \mu_{theoretical})^2 \quad (29)$$

where $\mu_{experimental}$ and $\mu_{theoretical}$ are the specific growth rates calculated from the experimental data and the mathematical models, respectively.

Chapter 4

4 Results and Discussion

4.1 Laboratory scale results

For each substrate, the fermentation process was characterized in terms of bacterial growth, pH, organic acids, and nutrient consumption. A comparison between the results obtained with different protocols was performed and, for each kinetic model proposed, the corresponding kinetic parameters were estimated. In this way, a validation of the changes applied to each protocol was obtained and proposal of new strategies to further optimize the process was possible. Moreover, a characterization of the dried products was performed to study the effect of microbial deactivation and the drying processes on the bacterial load and the desirable metabolites.

4.1.1 Rice flour results at laboratory scale

Pretreatment

Glucose and starch content in the raw rice flour (RRF), the un-pretreated rice flour suspension (URFS) and the hydrolyzed rice flour suspension (HRFS) are reported in Table 4.1.

The glucose value for HRFS includes the glucose initially present in the raw rice flour (0.45 mg/g $_{flour}$), added glucose (66.67 mg/g $_{flour}$), and glucose resulting from the enzymatic hydrolysis of starch using amylase (28.28 mg/g $_{flour}$). It is possible to observe a hydrolyzed starch percentage of 82.68% and a glucose release of 3.67%, evaluated with respect to the total starch content.

Table 4.1. Starch and glucose content in raw rice flour (RRF), un-pre-treated rice flour suspension (URFS) and hydrolysed rice flour suspension (HRFS); $\Delta_{hydrolysis}$ of starch and glucose resulting from the enzymatic treatment as percent variation and evaluated with respect to the initial starch amount.

	RRF (mg/g $_{flour}$)	**URFS** (mg/g $_{flour}$)	**HRFS** (mg/g $_{flour}$)	$\lvert\Delta_{hydrolysis}\rvert$ (%)
Starch	770	770	133.33	82.68
Glucose	0.45	67.12	95.40	3.67

In Gallo et al. (2021), enzymatic pretreatment of a 52% w/v wheat flour aqueous suspension was carried out using amylase (0.017% w/v) at 59 °C for 30 min. In that case, a hydrolyzed starch percentage of 81.41%, similar to that obtained in this work, was achieved.

Fermentation

Laboratory rice fermentations were performed following the recipes and the protocols described in the paragraphs 3.1 and 3.3.1, respectively. Bacterial growth, lactic acid production and pH trend during rice flour fermentation, carried out without pH control (protocol RF1), are reported in Figure 4.1. Lag and exponential phases lasted, respectively, from 0 to 4 h and from 4 to 14 h; the stationary phase started at time t_{14} and lasted until the end of the process (t_{24}) at least, when no pH control was applied. In particular, the bacterial charge, starting from an initial value of $2.08 \times 10^6 \pm 6.78 \times 10^5$ CFU/mL at time t_4, reached the value of $1.43 \times 10^8 \pm 1.85 \times 10^7$ CFU/mL at the end of exponential phase (t_{14}) and remained approximately constant during the stationary phase, up to time t_{24}. As shown in Figure 4.1b, pH decreased from an initial value of 5.89 ± 0.02 to a final value of 3.54 ± 0.04.
As expected, the lactic acid curve reflected the decreasing trend of pH during the process: an increasing lactic acid production was observed after 8 h of process, reaching a maximum value of 1.987 ± 0.058 g/L after 24 h of fermentation.
Moreover, butyric, acetic and propionic acids were measured but no trace was found.

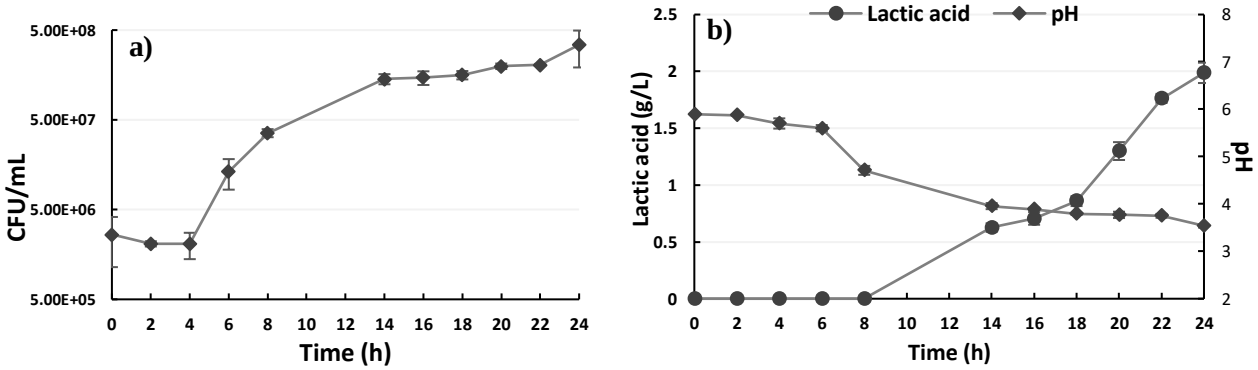

Figure 4.1. (a) Bacterial growth curve and (b) pH and lactic acid curves during rice flour fermentation carried out without pH control (protocol RF1) at laboratory scale.

When pH was controlled at 6.2, according to the protocol RF2, the growth curve (Figure 4.2) was characterized by a lag phase of 4 h, starting from a bacterial charge of $2.5 \times 10^6 \pm 8.5 \times 10^5$ CFU/mL after inoculation. The exponential phase lasted from 4 to 18 h of fermentation, reaching a maximum bacterial charge of $5.3 \times 10^8 \pm 1.47 \times 10^8$ CFU/mL at time t_{18}.
Lactic acid production curve is reported in Figure 4.3.

A maximum concentration of 5.4 ± 0.20 g/L was reached after 24 h of fermentation when pH was controlled and a doubling of lactic acid amount in the fermenting medium was observed, in comparison with the lactic acid production obtained in absence of pH control

Also, in this case, secondary acids (butyric, acetic and propionic acids) were not detected during the process, confirming absence of contamination.

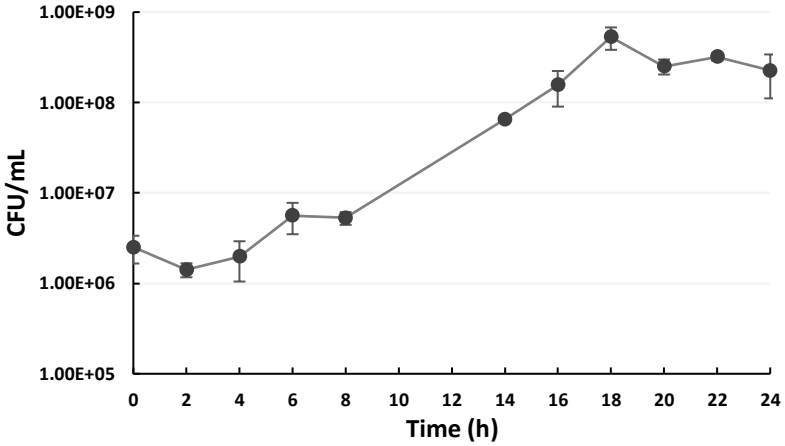

Figure 4.2. Bacterial growth curve during rice flour fermentation carried out with pH control (protocol RF2) at laboratory scale.

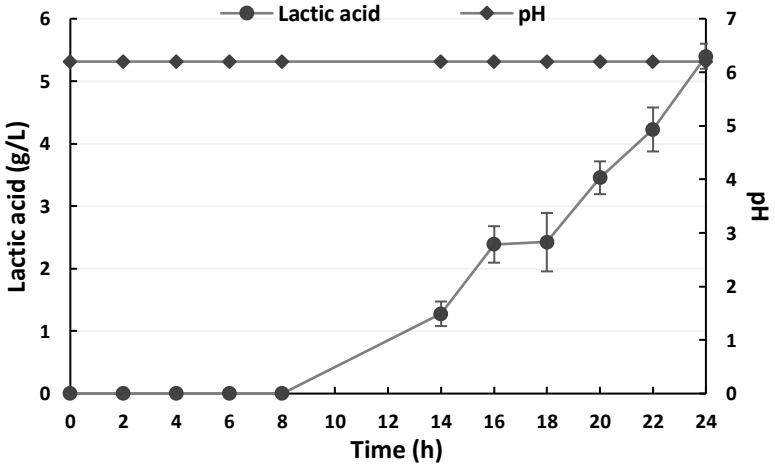

Figure 4.3. Lactic acid production during rice flour fermentation carried out with pH control (protocol RF2) at laboratory scale.

As shown in Table 4.2, when pH control was performed, fermentation tests provided higher values of the generation number, n, and the constant growth rate, k (8.52 and 0.71 h^{-1}, respectively), thus a lower doubling time t_d of 1.40 h in comparison with the values of 6.58, 0.66 h^{-1}, and 1.52 h, calculated for n, k, and t_d, respectively, in the absence of pH control.

These results indicated the better duplication capacity of the microorganism during the growth exponential phase, when pH of the rice medium was controlled at the optimal value for the microorganism's growth, throughout the entire period of fermentation.

Table 4.2. Fermentation parameters evaluated for rice flour fermentation with and without pH control: doubling time (t_d); constant growth rate (k); generation number (n); lag phase duration (t_{lag}); exponential phase duration (t_{exp}).

Protocol	**Process conditions**	**t_d** (h)	**k** (h^{-1})	**n**	**t_{lag}** (h)	**t_{exp}** (h)
RF1	Without pH control	1.52	0.66	6.58	4	10
RF2	With pH control	1.40	0.71	8.52	4	12

Moreover, for the optimized protocol RF2 (with pH control) the production of the functional metabolite was evaluated during the fermentation time. As shown in Figure 4.4, its content in the fermenting rice began to increase after 8 h of process, as well as for lactic acid, reaching an approximately constant value of 17 mg/L at time t_{18}, when the growth stationary period started.

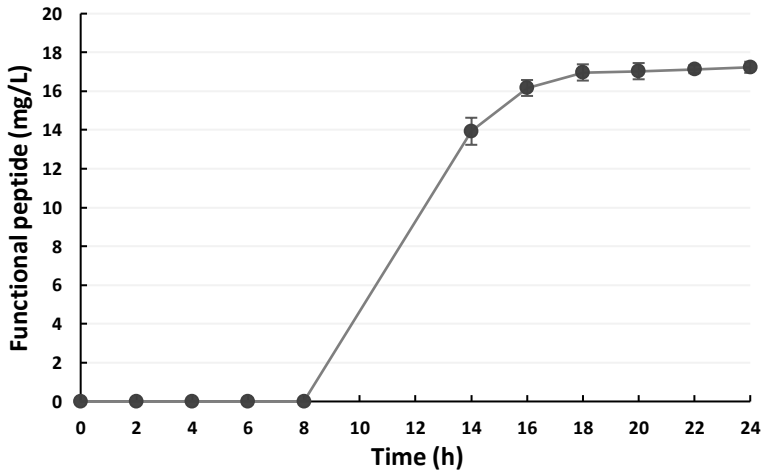

Figure 4.4. Functional metabolite production during rice flour fermentation carried out with pH control (protocol RF2) at laboratory scale.

Analysis of sugar consumption, observed during the process at the optimal pH, was performed and glucose, fructose and sucrose reduction were evaluated.

The consumption trends are reported in Figure 4.5: sucrose remained constant during the entire process (with an initial concentration of 4.88 g/L); fructose (initial concentration of 3.20 ± 0.05 g/L) was completely consumed, while glucose from an initial concentration of 14.30 ± 0.07 g/L dropped to 3.30 ± 0.03 g/L after 24 h of fermentation.

A glucose consumption of around 77% was observed while the starch concentration remained constant at a value of 20 g/L during the process, indicating that the glucose amount inside the hydrolysed rice suspension can be considered sufficient to satisfy the nutritional requirements of the microorganism.

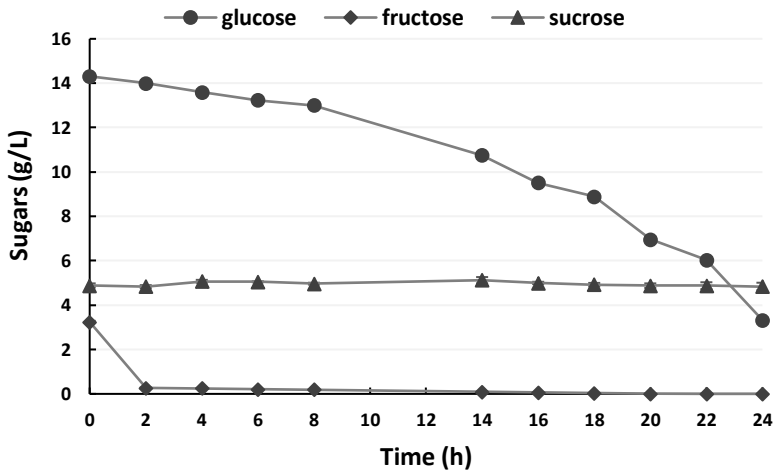

Figure 4.5. Sugar consumption (glucose, fructose and sucrose) during rice flour fermentation process, carried out with pH control (protocol RF2) at laboratory scale.

Fermentation kinetics

As reported in Figure 4.6, the specific growth rate μ, evaluated for the process with pH control, decreased during the process. This growth rate behaviour could be associated with the substrate consumption that occurred during the fermentation time or with a possible substrate/product inhibition.

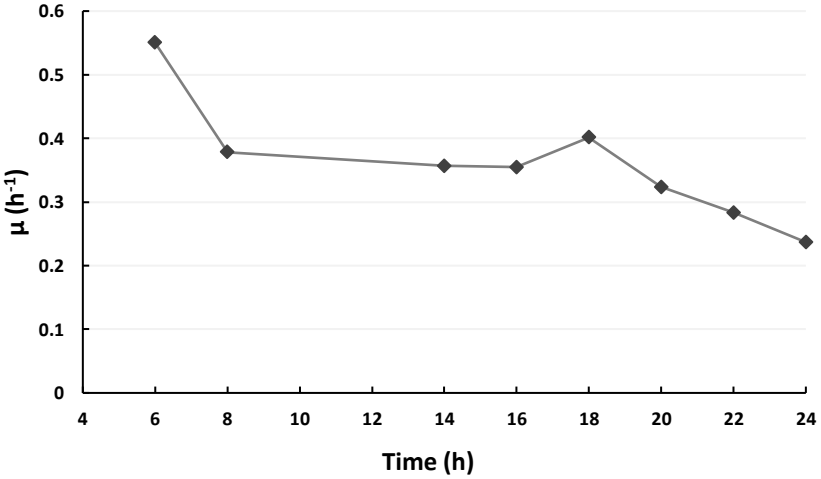

Figure 4.6. Specific growth rate trend during rice flour fermentation process carried out with pH control at laboratory scale.

The biokinetic parameters evaluated for the Monod model, the Haldane model, the product inhibition model, and the Luedeking−Piret model applied for both lactic acid and functional peptide were calculated, according to Equations (26), (27), (28), and (14), respectively.

In particular, the estimated μ_{max} (maximum specific growth rate), ks_1, ks_2 (semi-saturation constants for glucose and fructose, respectively), ki_1, ki_2 (substrate inhibition constants for glucose and fructose, respectively), k_p (product inhibition constant), α (growth-linked constant) and β (non-growth-linked constant) are reported in Table 4.3.

Table 4.3. The growth kinetic constants of Monod, Haldane, product inhibition, and Luedeking-Piret models (μ_{max}: maximum specific growth rate; ks_1, ks_2: semi-saturation constants for glucose and fructose respectively; ki_1, ki_2: substrate inhibition constants for glucose and fructose respectively; k_p: product inhibition constant; α: growth-linked constant; β: non-growth-linked constant), RSS, and the corresponding correlation coefficient (R^2).

Model	**μ_{max}** (h^{-1})	**ks_1** (g/L)	**ks_2** (g/L)	**k_{i1}** (g/L)	**k_{I2}** (g/L)	**K_p** (g/L)	**α** (g/g)	**β** (g/g h)	**α/β**	**RSS**	**R^2**
Monod	0.416	2.208	0.00065	-	-	-				0.00155	0.977
Haldane	0.324	11.613	0.00067	100	1.425	-				0.00133	0.980
Product inhibition	0.275	3.203	0.00048	-	-	131.163				0.00157	0.976
Luedeking−Piret (lactic acid)	-	-	-	-	-	-	0.023	1.029	0.022	0.0036	0.836
Luedeking−Piret (functional peptide)							0.005	0.0001	50	2×10^{-6}	0.840

- *Monod model:* semi-saturation constant for glucose ks_1 (2.208 g/L) was one order of magnitude lower than the glucose concentration during the exponential phase. Moreover, fructose concentration was much higher than ks_2 (0.00065 g/L). For these reasons, the amount of both substrates provided a specific growth rate surely higher than the half of the maximum growth rate, μ_{max} (0.42 h^{-1}), and satisfied widely the nutritional requirement for the microorganism growth.
- *Haldane model:* the value obtained for the semi-saturation constant ks_1 estimated for glucose was comparable with the initial glucose concentration, indicating that the glucose content in the medium provided a specific growth rate at least close to half of the maximum specific growth rate. Moreover, the semi-saturation constant for fructose, ks_2 (0.00065 g/L), was three orders of

magnitude lower than fructose concentration, which thus did not limit the microorganism growth. The glucose inhibition constant ki_1 (100 g/L) was comparable to the square of glucose amount while the fructose inhibition constant, ki_2 (1.425 g/L) was much higher than the square of fructose concentration. For these reasons, glucose and fructose did not provide inhibition on the microorganism growth rate, as fermentation proceeded.

- *Product inhibition*: the amounts of glucose and fructose in the fermenting medium provided a specific growth rate surely higher than the half of the maximum growth rate, having ks_1 and ks_2 equal to 3.203 g/L and 0.00048 g/L, respectively. Moreover, the product inhibition constant was 131.16 g/L, higher than the maximum lactic acid concentration (4.53 g/L) obtained after 24 h of fermentation. For these reasons, the increasing lactic acid concentration in the fermenting medium did not affect the bacterial growth rate.

- *Luedeking-Piret model for lactic acid:* as expected, the lactic acid production rate was mainly non-growth linked, as $\alpha/\beta = 0.022 << 1$. In fact, the lactic acid began to increase after 8 h, reaching its highest concentration during the stationary phase. The Luedeking model fitted the experimental lactic acid production rate with low accuracy ($R^2 = 0.836$).

- *Luedeking-Piret model for functional peptide:* as expected, the functional peptide production rate is growth linked, having $\alpha/\beta = 50 >> 1$. As well as for lactic acid, the Luedeking–Piret model fitted the experimental data concerning the functional peptide production rate with low accuracy ($R^2= 0.840$).

Deactivation and drying

A characterization in terms of microbial load, lactic acid, and functional metabolite concentration was done on stabilized rice fermented products (heat treated and dried) produced at lab scale, to evaluate the effect that two different drying technologies, freeze-drying and spray-drying, had on the main indicators of the fermentation process (bacterial charge and lactic acid) and on the functional compound of interest. Due to the heat treatment (90°C for 1 min), no bacterial charge was detected, as expected. As shown in Table 4.4, lactic acid concentration values in freeze dried and spray dried samples were comparable and not significantly different ($p > 0.05$), meaning that the two different drying methodologies had not a different impact on the product.

Moreover, the slight lactic acid reduction observed in dried samples when compared with fermented samples before the stabilizing treatments, could be mainly due to the heat treatment but statistically significant differences were not found. Also, functional peptide concentration was not significantly affected by heat treatment and both drying processes.

Table 4.4. Bacterial charge, lactic acid and functional peptide content in fermented rice products, stabilized by freeze-drying and spray drying at laboratory scale. The values marked with the same lowercase letters in the same column are not significantly different ($p > 0.05$).

Sample	**Bacterial charge** (CFU/mL)	**Lactic acid** (g/L)*	**Functional peptide** (mg/L)*
End of fermentation	8 logs	5.4 ± 0.20^{a}	17.23 ± 0.28^{b}
Heat treated & freeze-dried	< 2 logs	4.6 ± 0.4^{a}	18.11 ± 0.76^{b}
Heat treated & spray-dried	< logs	4.8 ± 0.3^{a}	18.40 ± 0.92^{b}

*Lactic acid and functional peptide concentrations reported for freeze and spray-dried rice powders were referred to the rehydration volume (1:15 w/v), used for reproducing the solid to water ratio of the fermentation batch.

4.1.2 Milk results at laboratory scale

Fermentation

Laboratory milk fermentations were performed following the recipes and the protocols described in the paragraphs 3.1 and 3.3.2, respectively. Bacterial growth, lactic acid production and pH trend during milk fermentation, carried out without pH control (protocol MF1), are reported in Figure 4.7. After a lag phase of 2 h, the bacterial charge, starting from an initial value of $1.57 \times 10^{6} \pm 1.96 \times 10^{5}$ CFU/mL at time t_2, reached the value of $2.23 \times 10^{8} \pm 2.66 \times 10^{7}$ CFU/mL at the end of exponential phase (t_{18}) and remained approximately constant during the stationary phase, up to time t_{24}.

As observed in Figure 4.7b, the production of lactic acid started after 2 h of fermentation, reaching a maximum value of 3.62 ± 0.21 g/L at time t_{22}. Moreover, pH decreased from an initial value of 6.60 ± 0.03 to a final value of 5.44 ± 0.06. Secondary acids (butyric, acetic and propionic acids) were not detected.

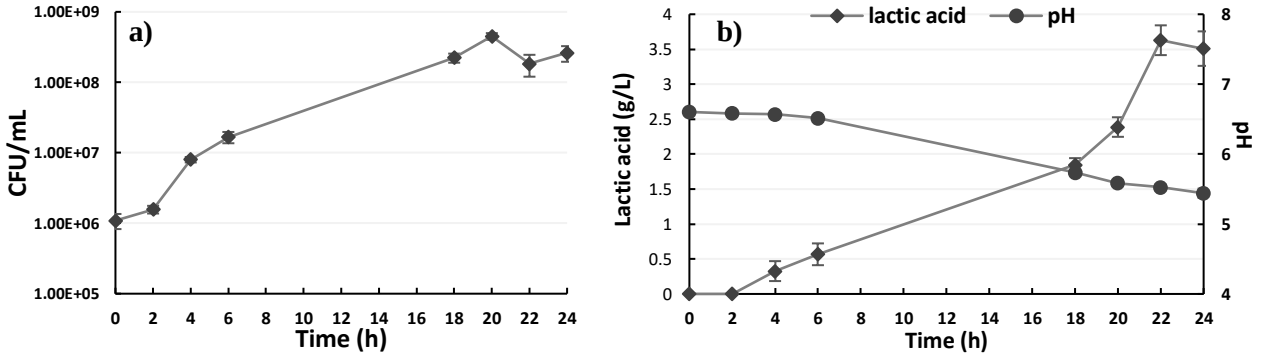

Figure 4.7. (a) Bacterial growth curve and (b) pH and lactic acid curves during milk fermentation carried out without pH control (protocol MF1) at laboratory scale.

When pH was controlled during the process (protocol MF2), the growth curve (Figure 4.8) was characterized by a lag phase of 4 h, starting with a bacterial charge of $2.40 \times 10^6 \pm 2 \times 10^5$ CFU/mL after inoculation, an exponential phase from time t_4 to time t_{14}, during which a final bacterial charge of $1.01 \times 10^8 \pm 1.2 \times 10^8$ CFU/mL was reached at time t_{14}, and a stationary phase that lasted from time t_{14} to the end of the process, maintaining a microbial load of 8 logs up to t_{24}.

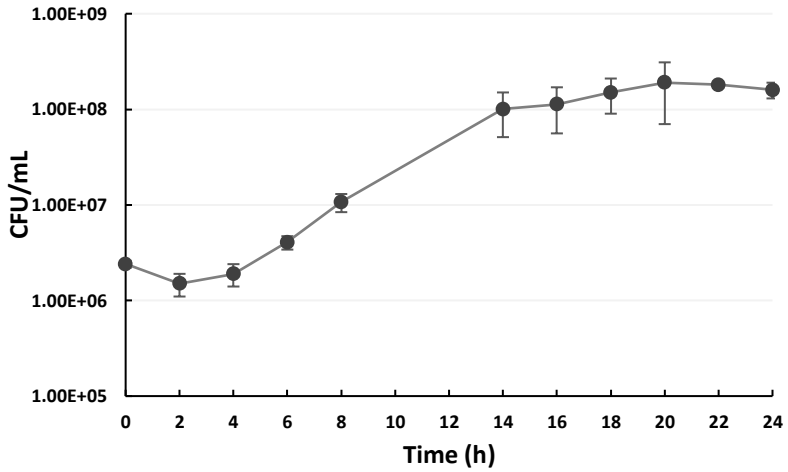

Figure 4.8. Bacterial growth curve during milk fermentation carried out with pH control (protocol MF2) at laboratory scale.

Lactic acid production curve is reported in Figure 4.9. The production of lactic acid started after 8 h of process, reaching a maximum amount of 1.2 ± 0.3 g/L after 24 h of fermentation.

While for rice the pH control allowed a higher production of lactic acid than that obtained without controlling pH, in milk a lower amount was observed when pH control was implemented.

Butyric, acetic and propionic acids were also measured but no traces were found, confirming the absence of contaminants in the medium.

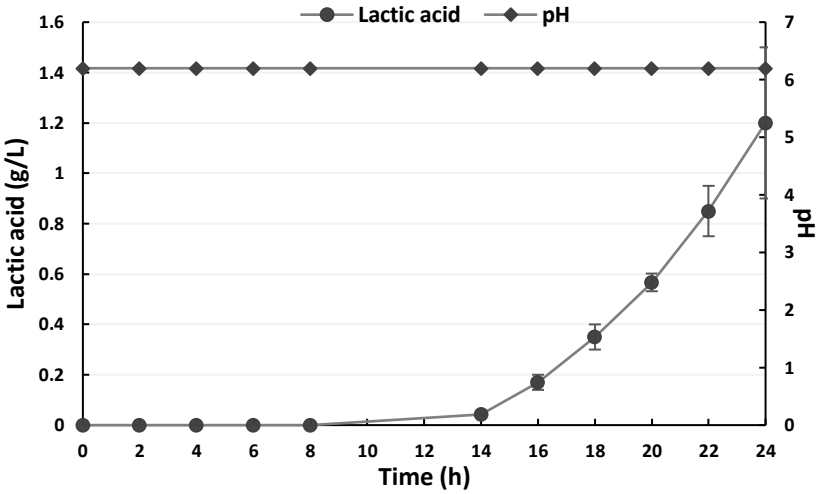

Figure 4.9. Lactic acid production during milk fermentation, carried out with pH control (protocol MF2) at laboratory scale.

Protocol MF2 provided best results in terms of growth kinetics, as confirmed by a shorter exponential phase time (10 h) than that observed for the protocol MF1 (16 h) and by the fermentation parameters reported in Table 4.5. In particular, fermentation tests carried out with pH control provided a slightly higher constant growth rate k of 0.53 h^{-1} and, thus, a lower doubling time t_d of 1.87 h in comparison with the values of 0.50 h^{-1}, and 1.97 h, calculated for k and t_d, respectively, in the absence of pH control. Also for milk, a better duplication capacity of the microorganism during the growth exponential phase was observed when pH was controlled at the optimal value of 6.2, although the results obtained during rice flour fermentation with both RF1 and RF2 in terms of doubling time and constant growth rate were better, as discussed in the paragraph 4.1.1.

Table 4.5. Fermentation parameters evaluated for milk fermentation with and without pH control: doubling time (t_d); constant growth rate (k); generation number (n).

Protocol	**Process conditions**	**t_d** (h)	**k** (h^{-1})	**n**	**t_{lag}** (h)	**t_{exp}** (h)
MF1	Without pH control	1.97	0.50	8.11	2	16
MF2	With pH control	1.87	0.53	5.35	4	10

As well as for rice, the production of the functional peptide was evaluated during milk fermentation process carried out with pH control (Figure 4.10). The peptide concentration began to be produced after 14 h of process, and increased during the entire stationary period of the microorganism.
A maximum concentration of 6.93 ± 0.5 mg/L was reached at time t_{24}.

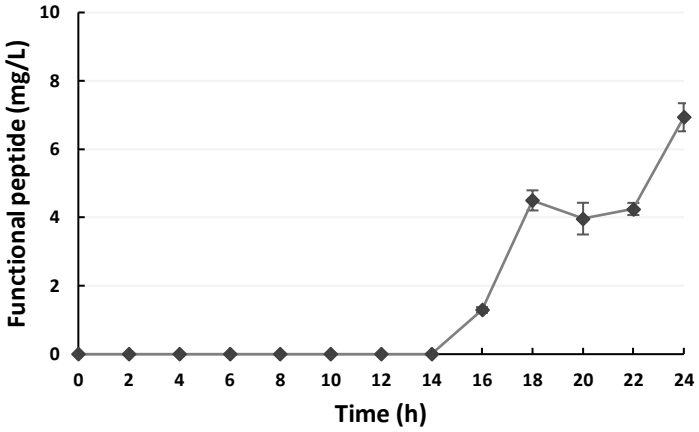

Figure 4.10. Functional metabolite production during milk fermentation carried out with pH control (protocol MF2) at laboratory scale.

Analysis on lactose, glucose, fructose and sucrose content in the fermenting milk during the process time (protocol MF2) was performed. Sucrose and fructose amounts were not detected; lactose concentrations remained approximately constant during the process (38.5 g/L), while glucose concentration decreased from an initial value of 23.7 ± 0.57 g/L to 17.3 ± 0.83 g/L after 24 h of fermentation, as shown in Figure 4.11.

Therefore, it was possible to assume glucose as the main microorganism' carbon source in milk.

A total glucose consumption of around 27% was observed in 24 h of process, suggesting that addition of 2% glucose, as reported in the fermentation recipe described in the paragraph 3.1, was probably excessive, as also confirmed by the kinetic analysis discussed in the next paragraphs.

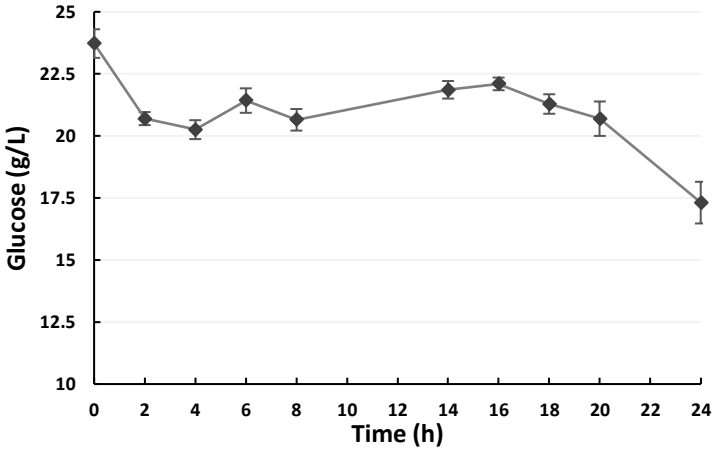

Figure 4.11. Glucose consumption during milk fermentation process carried out with pH control (protocol MF2) at laboratory scale.

Fermentation kinetics

For the reasons previously discussed, the kinetic analysis of milk fermentation process at laboratory scale was based solely on glucose, considered as the limiting substrate for the microorganism growth rate. In Figure 4.12, the decreasing specific growth rate trend during the fermentation process was reported and the biokinetic parameters present in Monod, Haldane, product inhibition, and Luedeking−Piret models were estimated (Table 4.6).

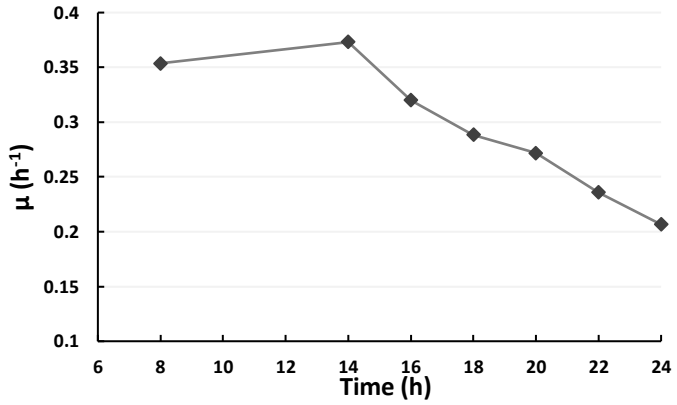

Figure 4.12. Specific growth rate trend during milk fermentation process carried out with pH control (protocol MF2) at laboratory scale.

Table 4.6. The growth kinetic constants of Monod, Haldane, product inhibition, and Luedeking-Piret models (μ_{max}: maximum specific growth rate; ks: semi-saturation constant; ki: substrate inhibition constant; k_p: product inhibition constant; α: growth-linked constant; β: non-growth-linked constant), RSS, and the corresponding correlation coefficient (R^2).

Model	**μ_{max}** (h^{-1})	**ks** (g/L)	**k_i** (g/L)	**k_p** (g/L)	**α** (g/g)	**β** (g/g h)	**α/β**	**RSS**	**R^2**
Monod	0.380	4.123	-	-	-	-	-	0.00656	0.999
Haldane	0.400	8.000	100	-	-	-	-	0.03144	0.904
Product inhibition	0.362	0.010	-	1.412	-	-	-	0.00076	0.920
Luedeking−Piret (lactic acid)	-	-	-	-	0.001	0.514	0.0019	0.00055	0.943
Luedeking−Piret (functional peptide)	-	-	-	-	0.005	4.216	0.0012	0.05119	0.983

- *Monod model* described with high accuracy the experimental specific growth rate ($R^2 = 0.999$), providing a maximum specific growth rate μ_{max} of 0.38 h^{-1} and a semi-saturation constant k_s of 4.123 g/L. Glucose amount in the fermenting medium during the exponential phase was much higher than the estimated k_s, indicating that glucose concentration was not limiting to microbial growth during the process.
- *Haldane model:* from the values calculated for $\mu_{max,}$ k_s and k_i (0.4 h^{-1}, 8 g/L and 100 g/L, respectively) it is possible to assert not only that glucose content did not limit the growth rate during the process but also that its concentration had an inhibiting effect on the specific growth rate in milk (being $k_I >> [glucose]^2$).

  This result and the low percent consumption of glucose (22%) discussed above, confirmed that the added glucose percentage in milk recipe could be reduced, as well as in rice, where 1% of added glucose was sufficient to satisfy the microorganism nutritional requirement. In this way, it could be possible to prevent its inhibition effect on the bacterial growth, ensuring at the same time a glucose concentration in the medium that does not limit the growth rate.
- *Product inhibition*: a lower value of k_s was calculated (0.01 g/L), meaning that glucose concentration in the fermenting medium was able to ensure a specific growth rate much higher than the half of the maximum growth rate. The value for the product inhibition constant k_p (1.412 g/L) was comparable with the lactic acid concentration in the medium, so the increasing lactic acid amount in the medium could have affected the bacterial growth kinetics.
- Luedeking-Piret model for lactic acid: as expected, the lactic acid production rate was mainly non-growth linked, as $\alpha/\beta = 0.00195 << 1$. The lactic acid began to increase after 14 h, when the stationary phase started, and reached its maximum concentration at time t_{24}.
- Luedeking-Piret model for functional peptide: as expected, the functional peptide production rate was non- growth linked, having $\alpha/\beta = 0.0012 << 1$. It followed the same behaviour of lactic acid formation, mainly developing during the growth stationary period.

Deactivation and drying

A characterization in terms of microbial load, lactic acid, and functional metabolite concentration was done on stabilized milk fermented products (heat treated and dried) produced at lab scale, to evaluate the effect that freeze-drying and spray-drying had on the main indicators of the fermentation process

(bacterial charge and lactic acid) and on the functional peptide. Due to the heat treatment (90°C for 1 min), no bacterial charge was detected, as expected.

As shown in Table 4.7, there were no statistically significant differences between lactic acid and peptide concentration values calculated for freeze dried and spray dried samples ($p > 0.05$), meaning that spray drying technology allowed to reach similar results to those obtained with the milder freeze-drying treatment.

Table 4.7. Bacterial charge, lactic acid and functional peptide content in fermented milk products, stabilized by freeze-drying and spray drying at laboratory scale. The values marked with the same lowercase letters in the same column are not significantly different ($p > 0.05$).

Sample	**Bacterial charge** (CFU/mL)	**Lactic acid** (g/L)*	**Functional peptide** (mg/L)*
End of fermentation	8 logs	1.2 ± 0.30^{a}	6.93 ± 0.50^{b}
Heat treated & freeze-dried	< 2 logs	0.92 ± 0.4^{a}	7.18 ± 0.12^{b}
Heat treated & spray-dried	< 2 logs	0.90 ± 0.3^{a}	7.20 ± 0.20^{b}

*Lactic acid and functional peptide concentrations reported for freeze and spray-dried milk powders were referred to the rehydration volume (1:10 w/v), used for reproducing the solid to water ratio of the fermentation batch.

4.1.3 Beans results at laboratory scale

Pretreatment

As discussed in paragraph 3.3.3, the preliminary pre-treatment protocols BT1 ÷ BT5 consisted of enzymatic hydrolysis and sterilization of a bean flour suspension, carried out at different temperatures/ times conditions.

In all cases, presence of contaminants (> 10^4 CFU/mL) in the fermenting medium was detected after 14 h of process. Probably, an inappropriate starch hydrolysis during the enzymatic pre-treatment and the resulting presence of a non-hydrolyzed starch fraction in the medium led to a too high viscosity of the suspension, with an inefficient heat conduction during the sterilization in autoclave and a non-homogeneous mixing during the fermentation process.

For these reasons, a completely different pretreatment was applied (protocol BT6), which started from whole beans, then processed by soaking, cooking and a subsequent size reduction operation.

The cooking phase allowed a preventive starch gelification and a first sterilization of the medium; then a strong autoclave cycle guaranteed a complete sterilization and absence of contamination.

Fermentation

Laboratory bean fermentations were performed following the recipes and the protocols described in the paragraphs 3.1 and 3.3.3, respectively. In particular, two mixing systems were used (I1 and I2) in order to observe their impact on the bacterial growth and the metabolite production.

The system I2 was designed after performing mixing tests with the impeller I1, using a food dye, which revealed the necessity to improve the mixing inside the bioreactor during the process.

Images taken from the mixing tests performed by using both impellers are shown in Figure 4.13: a different dye distribution in the medium was evident after 10s of mixing using I1 and I2 impellers.

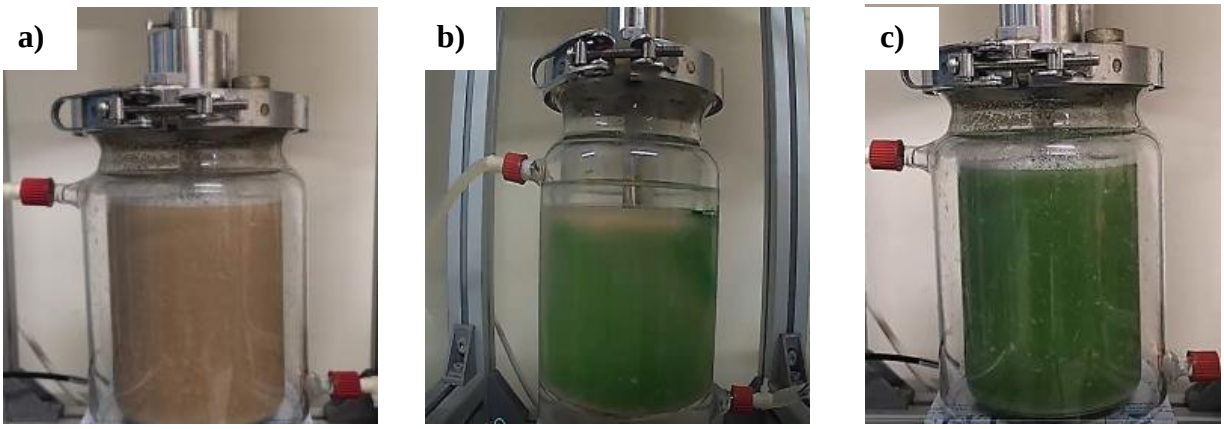

Figure 4.13. Images taken from mixing tests performed on the bean suspension, using a green dye: (a) bean suspension before adding the dye; dye distribution after 10s mixing performed by (b) impeller I1 and (c) impeller I2.

The bacterial growth curve of the fermentation process carried out using the mixing system I1 is shown in Figure 4.14a. It was characterized by a lag phase of 2 h, starting from an initial bacterial count of $2.83 \times 10^6 \pm 9.03 \times 10^5$ CFU/mL after inoculation, and an exponential phase from 2 h to 18 h. The stationary phase started at 18 h ending at 24 h of fermentation.

A maximum bacterial count of $1.06 \times 10^9 \pm 2.13 \times 10^8$ CFU/mL was reached at 20 h.

The pH decreased from an initial value of 6.09 ± 0.05 to a final value of 5.20 ± 0.06 and an increasing lactic acid production was observed, from an initial amount of 0.39 ± 0.029 g/L to a maximum value of 1.90 ± 0.058 g/L after 16 h of fermentation (Figure 4.14b). After 18 h, during the biomass stationary phase, the lactic acid concentration remained constant until the end of the process.

The lactic acid production trend reflected the slight decrease of pH during the process.

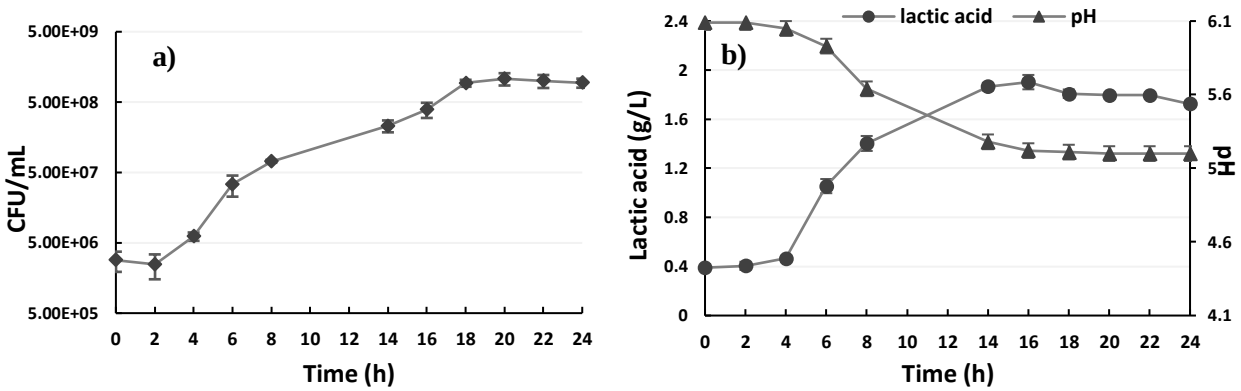

Figure 4.14. (a) Bacterial growth curve and (b) pH and lactic acid curves during bean fermentation carried out with impeller I1 (protocol BF1) at laboratory scale.

During the fermentation process of a cooked bean suspension, it was observed a consumption of glucose, fructose and total starch (Figure 4.15) while the sucrose content remained approximately constant at a concentration of 2.90 ± 0.06 g/L during the entire process time.

As observed in Figure 4.15, sugars reduction seemed to occur in a sequential manner during the process: starch concentration decreased during the first hours of the process; then the major glucose reduction was observed during the first exponential phase period and finally fructose was metabolized by the lactobacillus only during the last hours of the exponential phase.

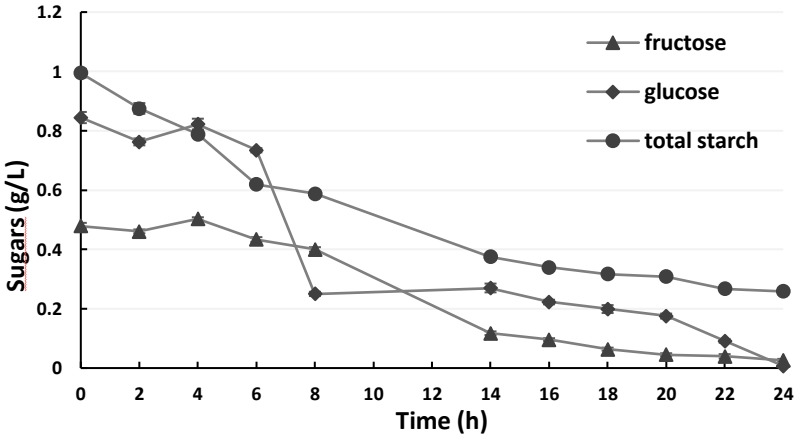

Figure 4.15. Sugar consumption (glucose, fructose and total starch) during bean fermentation carried out using the mixing system I1 (protocol BF1) at laboratory scale.

The bacterial growth, lactic acid production and sugar consumption curves obtained with protocol BF2 are shown in Figures 4.16, 4.17, and 4.18, respectively.

Bacterial curve was characterized by a lag phase of 2 h, starting with an initial microbial charge of $3.84 \times 10^6 \pm 3.58 \times 10^5$ CFU/mL, immediately after inoculation.

Growth exponential phase lasted 12 h, reaching the maximum value of $1.01 \times 10^9 \pm 2.05 \times 10^8$ CFU/mL at time t_{14}, which remained constant during the stationary phase of microorganism, until the end of the process (t_{24}). Moreover, also in this case, lactic acid concentration increased during the growth exponential phase and remained approximately constant during the stationary period (around 2 g/L).

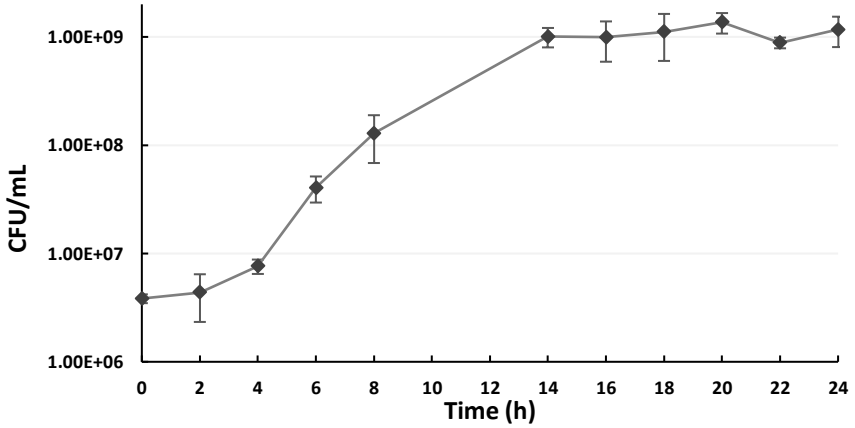

Figure 4.16. Bacterial growth curve during bean fermentation carried out with impeller I2 (protocol BF2) at laboratory scale.

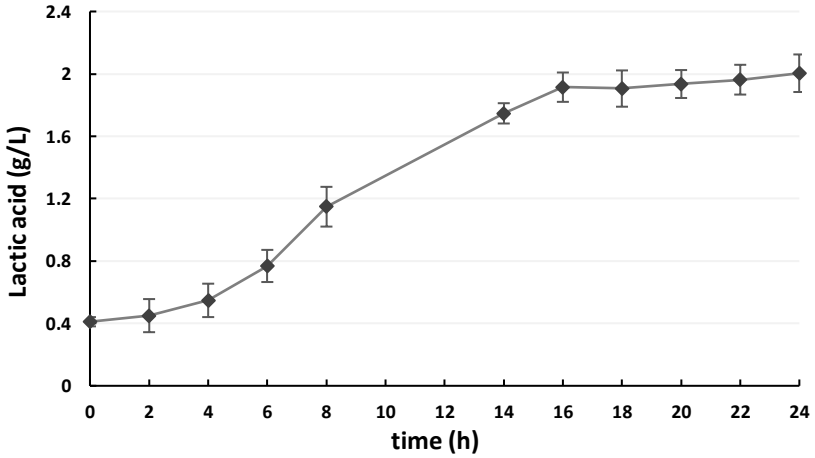

Figure 4.17. Lactic acid production during bean fermentation carried out with impeller I2 (protocol BF2) at laboratory scale.

As shown in Table 4.8, when mixing was improved using the system I2, fermentation tests provided a higher constant growth rate, k (0.65 h^{-1}) and, thus, a lower doubling time t_d of 1.53 h, in comparison with the values of 0.53 h^{-1}, and 1.87 h, calculated for k and t_d, respectively, using the mixing system I1. The faster achievement of the maximum values for microbial load and lactic acid produced was due to a major availability of nutrients in the fermenting medium, provided by the optimized mixing. This was confirmed by the sugar consumption curve, reported in Figure 4.18.

During the exponential phase, glucose and fructose were rapidly and completely consumed by the microorganism, while the total starch slightly decreased from 0.998 ± 0.005 g/L to 0.512 ± 0.002 g/L at the end of the process. Once the mixing was optimized, analysis on raffinose and stachyose content during the fermentation process were performed (Figure 4.19), with the aim to investigate the ability of the microorganism to consume even these complex sugars.

As observed, the initial raffinose and stachyose concentrations (0.56 ± 0.8 g/L and 10.87 ± 0.87 g/L, respectively) remained approximately constant during the entire process, meaning that LP CBA L74 was not able to consume these sugars during its metabolic activity.

Moreover, the functional peptide was not found in the fermenting medium until the end of the process, suggesting the necessity to optimize the fermentation protocol for this purpose, implementing pH control or glucose addition as well as for rice and milk.

Table 4.8. Fermentation parameters evaluated for bean fermentation carried out using two different mixing systems (I1 and I2): doubling time (t_d); constant growth rate (k); generation number (n); lag phase period (t_{lag}), and exponential phase period (t_{exp}).

Protocol	**Process conditions**	$\mathbf{t_d}$ (h)	**k** (h^{-1})	**n**	$\mathbf{t_{lag}}$ (h)	$\mathbf{t_{exp}}$ (h)
BF1	Mixing system I1	1.87	0.53	8.56	2	16
BF2	Mixing system I2	1.53	0.65	7.84	2	10

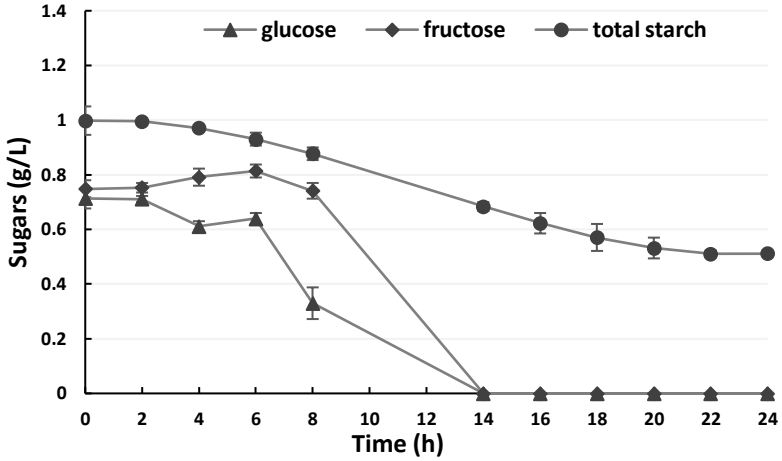

Figure 4.18. Sugar consumption (glucose, fructose and total starch) during bean fermentation carried out using the mixing system I2 (protocol BF2) at laboratory scale.

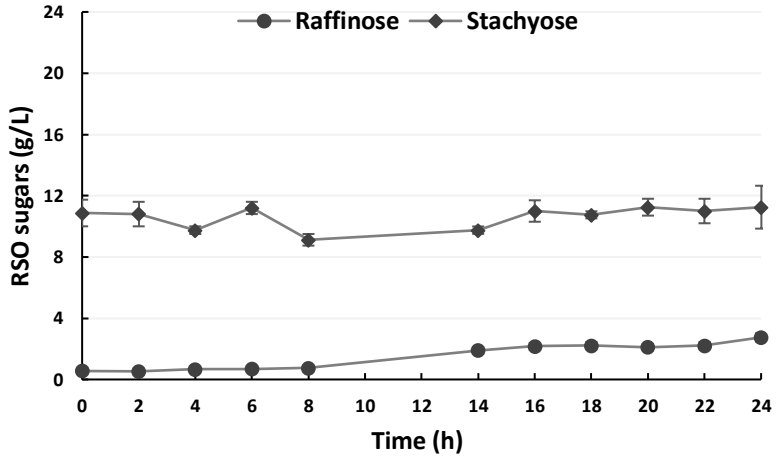

Figure 4.19. Raffinose and stachyose concentration trend during bean fermentation carried out using the mixing system I2 (protocol BF2) at laboratory scale.

Fermentation kinetics

As reported in Figure 4.20, the specific growth rate μ decreased during the fermentation process. The biokinetic parameters of Monod, Haldane, product inhibition and Luedeking–Piret models used for modelling growth rate and lactic acid production rate, were estimated (Table 4.9).

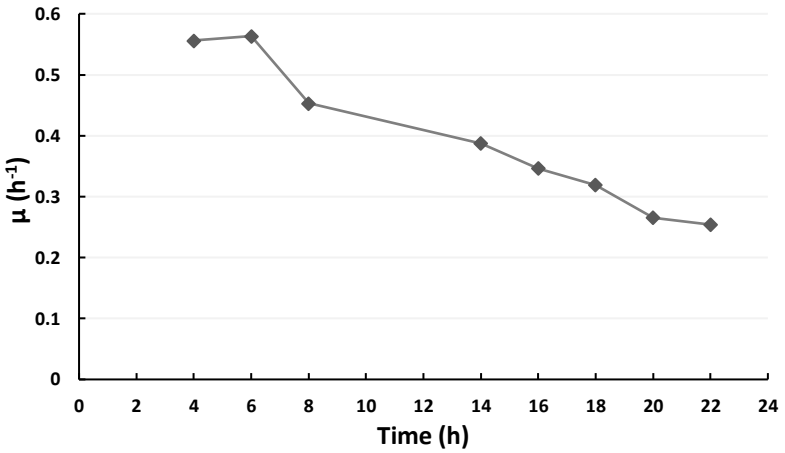

Figure 4.20. Specific growth rate trend during bean fermentation process carried out using the mixing system I2 (protocol BF2) at laboratory scale.

Table 4.9. The growth kinetic constants of Monod, Haldane, product inhibition, and Luedeking-Piret models (μ_{max}: maximum specific growth rate; ks_1, ks_2, ks_3: semi-saturation constants for starch, fructose, and glucose, respectively; ki_1, ki_2, ks_3: substrate inhibition constants for starch, fructose, and glucose, respectively; k_p: product inhibition constant; α: growth-linked constant; β: non-growth-linked constant), RSS, and the corresponding correlation coefficient R^2.

Model	**μ_{max}** (h^{-1})	**ks_1** (g/L)	**ks_2** (g/L)	**ks_2** (g/L)	**k_{i1}** (g/L)	**ki_2** (g/L)	**ki_3** (g/L)	**K_p** (g/L)	**α** (g/g)	**β** (g/g h)	**α/β**	**RSS**	**R^2**
Monod	0.452	0.004	0.317	20	-	-	-	-	-	-	-	1×10^{-5}	0.999
Haldane	0.770	0.005	0.164	11.19	0.987	1.502	0.981	-	-	-	-	1×10^{-11}	1
Product inhibition	0.456	0.002	0.339	20	-	-		225.78	-	-	-	0.00011	0.999
Luedeking–Piret	-	-	-		-	-		-	0.001	0.709	1×10^{-4}	0.87950	0.590

- *Monod model:* semi-saturation constants for starch and fructose, ks_1 (0.004 g/L) and ks_2 (0.317 g/L) respectively, was one order of magnitude lower than the starch and fructose concentration in the fermenting medium during the exponential phase. Conversely, glucose concentration was much lower than the corresponding semi-saturation constant estimated, ks_3 (20 g/L).
  For these reasons, glucose could be considered the main growth limiting substrate and its content could be sufficient to guarantee a specific growth rate at least close to half of the maximum specific growth rate.

- *Haldane model*: also, in this case, the estimated values for ks_1, ks_2, and ks_3 suggested glucose content to be limiting for the growth rate, having ks_3 (11.19 g/L) one order of magnitude higher than glucose amount in the medium.
  Moreover, the starch inhibition constant ki_1 (0.987 g/L) was comparable to the square of starch amount in the bean suspension, while the fructose and glucose inhibition constants, ki_2 (1.502 g/L) and ki_3 (0.981 g/L) were higher than the square of fructose and glucose concentrations, respectively. This suggested that glucose and fructose amounts did not provide inhibition on the microorganism growth rate.

- *Product inhibition:* the semi saturation constants related to starch, fructose and glucose, had similar values to those estimated by Monod and Haldane and the same considerations about the role of glucose as limiting carbon sources in the fermenting medium could be done.
  Moreover, the product inhibition constant was 225.78 g/L, higher than the maximum lactic acid concentration (2 g/L) obtained after 24 h of fermentation: the increasing lactic acid concentration in the fermenting medium did not affect the bacterial growth rate.

- *Luedeking-Piret model for lactic acid:* although the lactic acid production curve showed a mixed behaviour, growth and non-growth linked, as observed in Figure 4.17, the estimated parameters, α and β, indicated a non-growth linked trend of the lactic acid production rate ($\alpha/\beta << 1$).
  The Luedeking-Piret model fitted the experimental lactic acid production rate with low accuracy ($R^2 = 0.590$).

The kinetic study suggested that it could be possible to reach higher specific growth rate and improve the kinetic performances of bean fermentation by optimizing the content or the availability of the limiting substrates (glucose in this case) or by setting optimal growth conditions for the microorganism (optimal pH conditions for instance).

Deactivation and drying

Stabilized bean fermented products (heat treated and dried) produced at laboratory scale were characterized in terms of microbial load and lactic acid concentration, to evaluate the effect that freeze-drying and spray-drying have on the main indicators of the fermentation process.

Microbial inactivation protocols BI1÷ BI5, described in the paragraph 3.3.3, did not provide the desirable residual bacterial load (< 10^2 CFU/mL) inside the stabilized powder, while protocol BI6, consisting of autoclaving at 90°C for 20 min in glass bottle (ID 6.3 cm), ensured the required bacterial reduction. As shown in Table 4.10, after the thermal treatment, lactic acid concentration values in freeze dried and spray dried samples, were comparable and not significantly different ($p > 0.05$).

Table 4.10. Bacterial charge and lactic acid content in fermented bean products, stabilized by freeze-drying and spray drying at laboratory scale. The values marked with the same lowercase letters in the same column are not significantly different (p > 0.05).

Sample	**Bacterial charge** (CFU/mL)	**Lactic acid** (g/L) *
End of fermentation	9 logs	2.0 ± 0.12[a]
Heat treated & freeze-dried	< 2 logs	1.95 ± 0.05[a]
Heat treated & spray-dried	< 2 logs	1.97 ± 0.01[a]

*Lactic acid concentration reported for freeze and spray-dried bean powders was referred to the rehydration volume (1:10 w/v), used for reproducing the solid to water ratio of the fermentation batch.

4.1.4 Physical properties of fermented rice, milk and beans

Table 4.11 shows a comparison of water adsorption capacity, water adsorption index, oil holding capacity, and foam capacity (defined in the paragraph 3.4) calculated in raw rice, milk and bean powders and the corresponding fermented samples.

Rice fermentation led to a reduction of WAC and WAI (from 1.3 ± 0.09 mL/g and 2.41 ± 0.07 g/g, respectively to 0.42 ± 0.04 mL/g and 1.48 ± 0.06 g/g, respectively), while improved the oil binding capacity of the matrix (from 2.28 ± 0.08 mL/g to 5.20 ± 0.12 mL/g). No foam was observed for rice suspension. An opposite effect was registered on milk. WAC and WAI in raw milk (0.65 ± 0.03 mL/g and 1.65 ± 0.02 g/g respectively) were lower than the values determined after fermentation process (0.88 ± 0.04 mL/g and 1.88 ± 0.02 g/g, respectively), while a reduced capacity to adsorb oil was observed (from 5.75 ± 0.02 mL/g in raw milk to 3.39 ± 0.09 mL/g after fermentation).

Moreover, a slight reduction of the foam capacity was observed (from 10 ± 1.55% to 7.5 ± 0.36 %) but the difference cannot be considered statistically significant (t-student test provided $p > 0.05$).

For beans, both water and oil retention capacity decreased due to fermentation and a foam reduction from 30 ± 1.65% to 20 ± 1.30% due to the fermentation process was observed.

Table 4.11. Water adsorption capacity (WAC), water adsorption index (WAI), oil holding capacity, (OHC), and foam capacity (FC) evaluated for raw and fermented rice, milk, and bean samples. The values marked with different lowercase letters in the same column are significantly different ($p < 0.05$).

Substrate	**WAC** (mL/g)	**WAI** (g/g)	**OHC** (mL/g)	**FC** (%)
Raw rice flour	1.30 ± 0.09^{a}	2.41 ± 0.07^{a}	2.28 ± 0.08^{a}	-
Fermented rice flour	0.42 ± 0.04^{b}	1.48 ± 0.06^{b}	5.20 ± 0.12^{b}	-
Raw milk	0.65 ± 0.03^{c}	1.65 ± 0.02^{c}	5.75 ± 0.02^{c}	10.00 ± 1.55^{a}
Fermented milk	0.88 ± 0.04^{d}	1.88 ± 0.02^{d}	3.39 ± 0.09^{d}	7.50 ± 0.36^{a}
Raw beans	4.55 ± 0.03^{e}	5.53 ± 0.04^{e}	6.67 ± 0.12^{e}	30 ± 1.65^{b}
Fermented beans	3.90 ± 0.14^{f}	4.31 ± 0.13^{f}	3.11 ± 0.11^{f}	20 ± 1.30^{c}

4.2 Pilot scale results

Pilot scale trials were carried out in the "Kraft Heinz Innovation center" in Nijmegen, The Netherlands, reproducing the characterizations performed at laboratory scale.

Fermentation trials were carried out on rice flour and milk using a 200 L bioreactor with temperature control (at 37°C) and pH control (at 6.2), and using a more concentrated NaOH, necessary to buffer larger volumes (200 L) than those of the lab scale (1 L). At the end of the fermentation process, the fermented medium was inactivated inside the same bioreactor, bringing the system at 90°C and holding for 1 min. Spray drying was the only drying technology employed, taking into account that both drying procedures tested at lab scale, i.e., freeze drying and spray drying, led to comparable results. In addition, a shelf-life analysis was performed on the fermented and stabilized rice and milk samples obtained.

4.2.1 Rice flour results at pilot scale

Fermentation

Pilot rice fermentations were performed following the recipes and protocols described in the paragraph 3.1 and 3.5.1, respectively. The growth curve is reported in Figure 4.21.

It was characterized by a lag phase of less than 2 h, starting with a bacterial charge of $8.25 \times 10^5 \pm 1.76 \times 10^5$ CFU/mL after inoculation, an exponential phase that started almost immediately, up to 16 h of fermentation, reaching a maximum charge of $1.27 \times 10^9 \pm 6.9 \times 10^8$ CFU/mL, and a stationary phase from t_{16}, during which a bacterial load of about 9 logs was maintained until the end of the process (t_{24}).

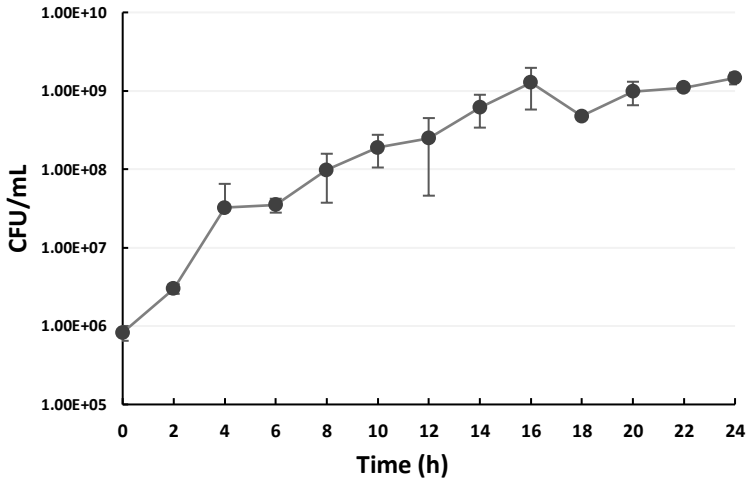

Figure 4.21. Bacterial growth curve during rice flour fermentation carried out at pilot scale.

Lactic acid and peptide production curves are reported in Figure 4.22 and 4.23, respectively.
The non-growth linked production of lactic acid, observed at laboratory scale, was confirmed also at pilot scale and a maximum concentration of 6.33 ± 0.70 g/L was reached after 24 h of process.
As for the functional peptide, it began to be detected after 12 h of fermentation and reached its maximum value of 22.60 ± 4.10 mg/L that remained approximately constant during the stationary phase, until the end of the process.

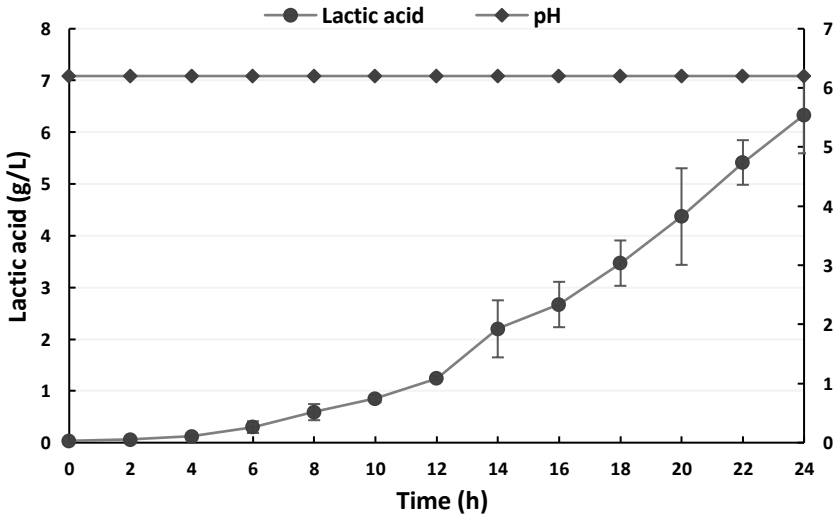

Figure 4.22. Lactic acid production curve during rice flour fermentation carried out at pilot scale.

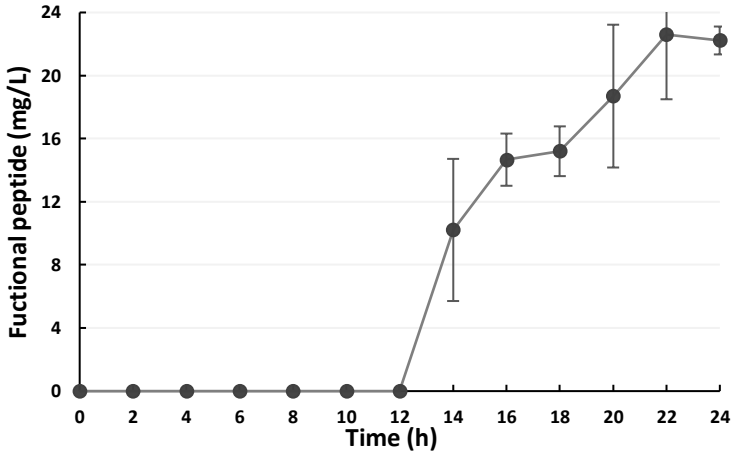

Figure 4.23. Functional peptide production curve during rice flour fermentation carried out at pilot scale.

Deactivation and drying

As reported in Table 4.12, a negligible bacterial load (< 10^2 CFU/mL) was detected after the heat treatment carried out on the fermented rice sample. Also, in this case, lactic acid and functional peptide concentrations were not affected by heating and spray drying processes. A low reduction was observed after the heat treatment but the difference was not statistically significant ($p > 0.05$).

Table 4.12. Bacterial charge, lactic acid and functional peptide content in fermented rice products, stabilized by spray drying at pilot scale. The values marked with the same lowercase letters in the same column are not significantly different ($p > 0.05$).

Sample	**Bacterial charge** (CFU/mL)	**Lactic acid** (g/L)*	**Functional peptide** (mg/L)*
End of fermentation	9 logs	6.33 ± 0.74^a	22.22 ± 0.88^b
Heat treated	< 2 logs	5.50 ± 0.57^a	21.70 ± 0.35^b
Heat treated & spray-dried	< 2 logs	5.20 ± 0.74^a	21.90 ± 0.30^b

*Lactic acid and functional peptide concentrations reported for heat treated and spray-dried rice powders were referred to the rehydration volume (1:15 w/v), used for reproducing the solid to water ratio of the fermentation batch.

4.2.2 Milk results at pilot scale

Fermentation

Pilot milk fermentations were performed following the recipes and protocols described in the paragraphs 3.1 and 3.5.2, respectively. The growth curve is reported in Figure 4.24.

It was characterized by a lag phase of 2 h, starting with a bacterial charge of $1.12 \times 10^6 \pm 7.42 \times 10^5$ CFU/mL, an exponential phase from 2 to 14 h, reaching a maximum load of $6.70 \times 10^8 \pm 3.75 \times 10^8$ CFU/mL at 20 h of fermentation, and a stationary phase from time t_{14} to the end of the process, during which a charge of 8 logs was maintained.

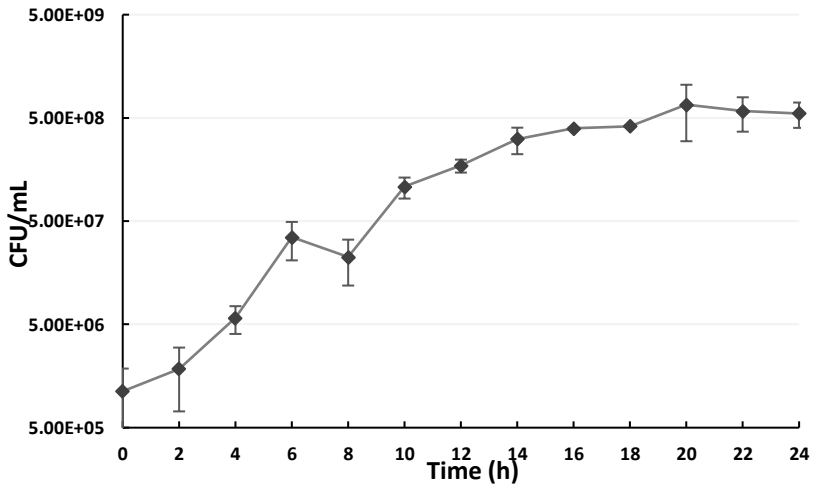

Figure 4.24. Bacterial growth curve during milk fermentation carried out at pilot scale.

Lactic acid and peptide production curves are reported in Figure 4.25 and 4.26, respectively.
As for lactic acid, the non-growth linked production trend observed at laboratory scale, was confirmed also at pilot scale and a maximum concentration of 2.1 ± 0.25 g/L was reached after 24 h of process.
The functional peptide content began to be detected in the medium after 14 h of fermentation process, showing a non-growth-linked trend, as occurred during fermentation at laboratory scale.
It reached its maximum concentration of 14.2 ± 0.8 mg/L at the end of the process (at time t_{24}).

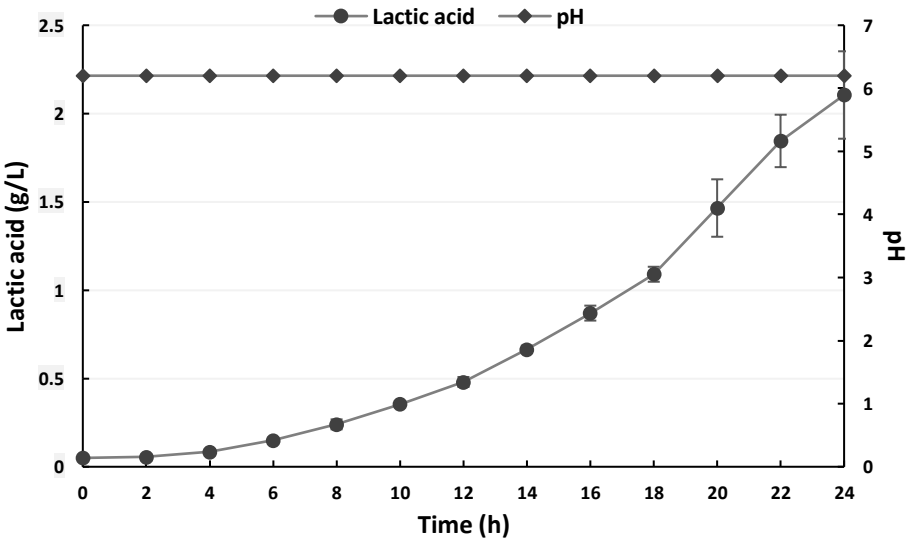

Figure 4.25. Lactic acid production curve during milk fermentation carried out at pilot scale.

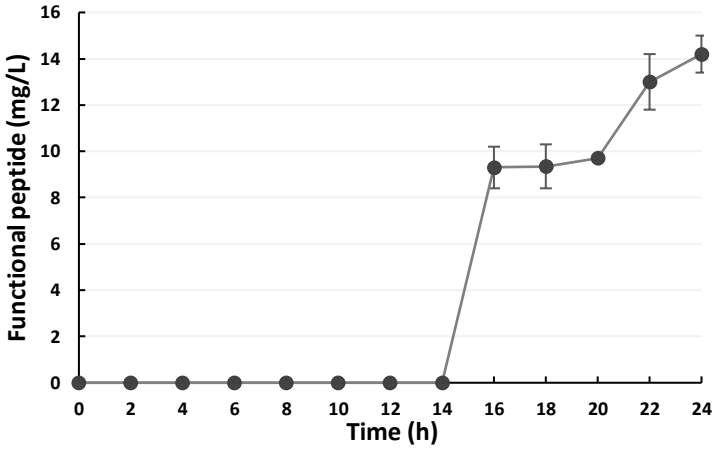

Figure 4.26. Functional peptide production curve during milk fermentation carried out at pilot scale.

Deactivation and drying

As reported in Table 4.13, a negligible bacterial load (< 10^2 CFU/mL) was detected after the heat treatment. Moreover, lactic acid and functional peptide concentrations were not affected by heating and spray drying process. A low reduction was observed after heating but the difference was not statistically significant ($p > 0.05$).

Table 4.13. Bacterial charge, lactic acid and functional peptide content in fermented milk products, stabilized by spray drying at pilot scale. The values marked with the same lowercase letters in the same column are not significantly different (p > 0.05).

Sample	**Bacterial charge** (CFU/mL)	**Lactic acid** (g/L)*	**Functional peptide** (mg/L)*
End of fermentation	8 logs	2.1 ± 0.25[a]	14.2 ± 0.80[b]
Heat treated	< 2 logs	1.90 ± 0.20[a]	13.70 ± 0.36[b]
Heat treated & spray-dried	< 2 logs	1.90 ± 0.23[a]	13.90 ± 0.51[b]

*Lactic acid and functional peptide concentrations reported for heat treated and spray-dried milk powders were referred to the rehydration volume (1:10 w/v), used for reproducing the solid to water ratio of the fermentation batch.

4.3 Shelf-life analysis

Shelf-life analysis was carried out on milk and rice fermented powders produced at pilot scale.

The analysis was performed for six months; the products were stored at three different temperatures, namely 4°C, 20°C and 37°C. Each product was characterised in term of organoleptic properties (texture, appearance-colour, and taste), *Lactobacillus paracasei* CBA L74's load and lactic acid concentration.

The sensory evaluation forms were filled in by expert panellists (some original comments have been reported) for both fermented milk and rice powders (Tables 4.14 and 4.15, respectively).
The microbiological and chemical characterizations performed on milk and rice powders are reported in Tables 4.16 and 4.17, respectively. Before each panel test, the presence of aerobic and anaerobic moulds in milk and rice fermented powder was evaluated by company QA, resulting in safe (data not shown). From the sensory evaluation form of fermented milk powder (Table 4.14) it is clear that after three months, milk powder stored at 37°C started to change its organoleptic properties, acquiring an unacceptable taste and appearance; the products stored at 4 and 20 °C resulted more stable, with slight changes over five months in terms of taste and appearance.
As regards the rice fermented powder, the situation seemed to be more stable, as shown in Table 4.15, since only after five months the sensory properties started to change for all storage temperatures, but in a negligible way.

Table 4.14. Filled sensory evaluation form of fermented milk powder produced at pilot scale.

Date	**4°C**	**20°C**	**37°C**	**Conclusion**
Month 0	n.a.		n.a.	4 bags (2 bags from the same batch seemed different for 2 panellists)
Month 1			Seems flatter in taste less dairy	No significant difference
Month 2			Colour: More yellow powder Taste: Slight cheesy off-taste	Order correctly identified, light off-flavour at 37°C, borderline
Month 3	Most neutral milky	Colour: no significant difference Taste: less clean acceptable	Colour: Very yellow Taste: Off flavour, cheesy and caramelised	Order identified correctly, 37°C not acceptable
Month 5		Colour: no difference Taste: slight oxidized less fresh	Colour: Very brown powder, caramelized colour also dissolved Taste: Off odour rancid, oxidized, caramelised	Order identified correctly, 37°C not acceptable

Table 4.15. Filled sensory evaluation form of fermented rice powder produced at pilot scale.

Date	4°C	20°C	37°C	Conclusion
Month 1	n.a.	Begin / middle / end	n.a.	Powder looks agglomerated but disperse when spread with the spoon. Fairly good dissolvability in water. No taste difference.
Month 2				Powder same appearance. No difference.
Month 3		Flatter, less cereal taste	Cardboard taste	No significant differences
Month 4		Slight	Slight metallic, cardboard taste	Colour: all the same Taste: Slight but no significant differences.
Month 5	Lower rice taste	Slight cardboard	Slight cardboard	Colour: all the same Taste: Slight but negligible difference.
Month 6	Very low rice taste		Slight cardboard	Colour: similar Taste: Slight but negligible difference

In Table 4.16, data from microbiological (LP CBA L74) and chemical (lactic acid and functional peptide concentrations) characterizations of milk fermented powder over time are reported.

During all five months no bacterial load was detected, as expected, since the product was inactivated and dried before storage. As regard lactic acid concentration, a reduction of around 50% was recorded after five months of storage (from an initial value of 2.5 g/L the concentration decreased up to 1.2 g/L after five months at 20°C) while the peptide amount slightly decreased from an initial value of 10.8 mg/L to 8.8 mg/L after five months of storage at 20°C. Lactic acid and peptide concentrations were more or less the same for products stored at the three different temperatures, suggesting that probably the reduction was not due to the temperature but only to the storage timing.

In Table 4.17, a characterization, over time and at different storage temperatures, in terms of microbial load, lactic acid, and functional peptide concentrations of rice fermented powder is reported.

Also, in this case no bacterial load was detected for the entire period of storage, as expected.

As regards the lactic acid concentration, after six months of storage a reduction of around 30% from the initial condition was detected: the lactic acid concentration in freshly produced samples was 5.5 g/L, reducing to 3.5 g/L after six months (20°C). Moreover, functional peptide concentration decreased from an initial content of 19.7 mg/L to 18.6 mg/L after six months of storage at 20°C.

Table 4.16 Microbial and chemical characterization of milk fermented powder produced at pilot scale.

Sample (name/ time/ temperature)	**Bacterial load** (CFU/mL)	**Lactic acid** (g/L)	**Functional peptide** (mg/L)
Milk 0 (immediately after production)	/	2.5	10.8
Milk 1 month 4°C	/	1.7	9
Milk 1 month 20°C	/	1.7	9
Milk 1 month 37°C	/	1.8	9.2
Milk 2 months 4°C	/	1.6	9.8
Milk 2 months 20°C	/	1.7	9.6
Milk 2 months 37°C	/	1.6	9.4
Milk 3 months 4°C	/	1.9	9.8
Milk 3 months 20°C	/	1.9	8.8
Milk 3 months 37°C	/	1.7	8.8
Milk 5 months 4°C	/	1.3	9.2
Milk 5 months 20°C	/	1.2	8.8
Milk 5 months 37°C	/	1.2	8.6

Table 4.17. Microbial and chemical characterization of rice fermented powder produced at pilot scale.

Sample (name/ time/ temperature)	**Bacterial load** (CFU/mL)	**Lactic acid** (g/L)	**Functional peptide** (mg/L)
Rice 0 (immediately after production)	/	5.5	19.7
Rice 1 month 4°C	/	4.7	19.1
Rice 1 month 20°C	/	4.9	19.2
Rice 1 month 37°C	/	5.8	20
Rice 2 months 4°C	/	5.9	19.2
Rice 2 months 20°C	/	6.2	19.7
Rice 2 months 37°C	/	5.6	18.2
Rice 3 months 4°C	/	5.5	16.5
Rice 3 months 20°C	/	5.7	16.2
Rice 3 months 37°C	/	5.3	15.9
Rice 4 months 4°C	/	5.5	18.9
Rice 4 months 20°C	/	5.4	16.9
Rice 4 months 37°C	/	5.3	16
Rice 6 months 4°C	/	3.8	19.2
Rice 6 months 20°C	/	3.5	18.6
Rice 6 months 37°C	/	3.9	18.6

It's clear for both fermented powders that changes in the organoleptic properties could be ascribed to changes in the main chemical compounds present in the products and that rice fermented powder seemed to be more stable than milk over time. It is necessary to continue the analysis up to 24 months to have a final idea.

4.4 Comparison between laboratory scale and pilot scale results

The scaling up of the processes resulted very efficient although the differences in terms of volume, apparatus and some preparation phases.

The comparison of the main results between lab and pilot scales is shown below. In any case, it is clear that rice fermentation, for both scales, gave better results in terms of bacterial growth and lactic acid production, probably for the rice prebiotic components.

4.4.1 Comparison of lab/pilot scale results for rice

Fermentation

As shown in Figure 4.27, bacterial growth curves for both scales followed approximately the same trend; the main differences were in the lag phase duration, that at lab scale lasted 4 h and at pilot scale less than 2 h, and in the maximum bacteria charge achieved: $5.3 \times 10^8 \pm 1.47 \times 10^8$ CFU/mL (t_{18}) at lab scale and $1.27 \times 10^9 \pm 6.9 \times 10^8$ CFU/mL (t_{16}) at pilot scale.

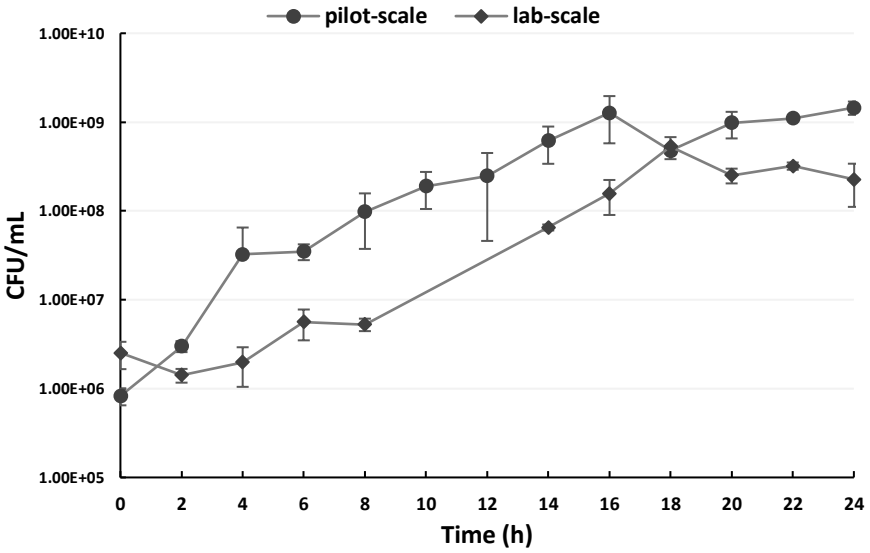

Figure 4.27. Comparison between laboratory (protocol RF2) and pilot scale rice growth curves.

Figure 4.28 and Figure 4.29 show the comparison between lab and pilot scale lactic acid and peptide production curves, respectively. As for lactic acid, the trend was the same with a final higher lactic acid production of 6.3 ± 0.7 g/L at pilot scale after 24 h of fermentation compared with 5.4 ± 0.20 g/L (t_{24}) at lab scale. Also, the functional peptide concentration at pilot scale reproduced the same trend of that observed at laboratory scale: in both cases, a plateau was reached after 14 h of fermentation corresponding to values of approximately 17 mg/L and 22 mg/L achieved in lab scale and pilot scale experimentations, respectively.

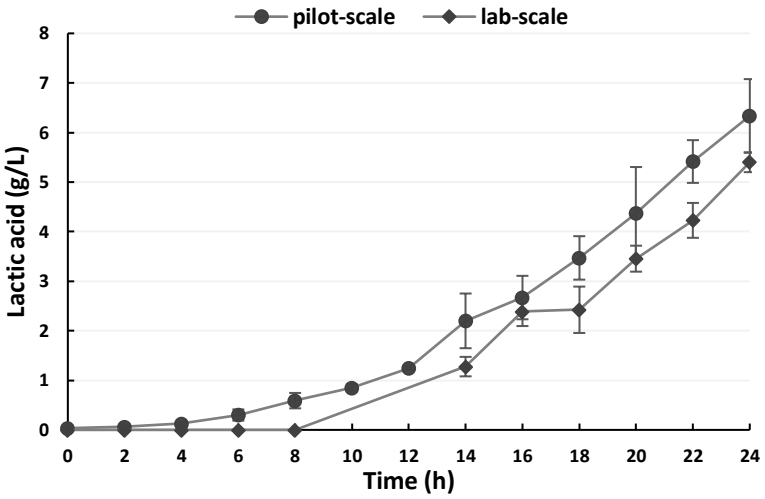

Figure 4.28. Comparison between laboratory (protocol RF2) and pilot scale rice lactic acid production curves.

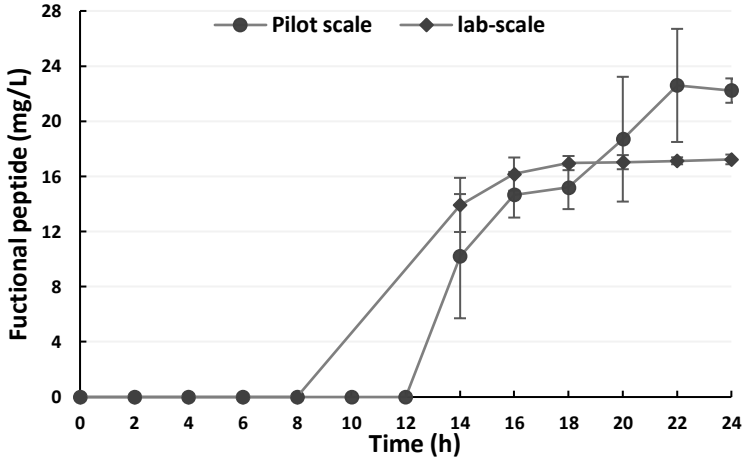

Figure 4.29. Comparison between laboratory (protocol RF2) and pilot scale rice functional peptide production curves.

Deactivation and drying

The stabilizing treatments (heat and drying treatments) on fermented rice flour had a low impact on the quality of the product that was confirmed for both scales, as reported in Table 4.18.

Statistically significant differences between lactic acid and peptide concentrations determined in lab and pilot scale trials were observed (both before and after stabilization).

Table 4.18. Comparison between bacterial growth, lactic acid, and functional peptide concentration in fermented rice, before and after stabilizing treatments performed at laboratory and pilot scale. The values marked with different lowercase letters in the same arrow are significantly different (t-student test provided $p < 0.05$).

Sample	**Bacterial charge** (CFU/mL)		**Lactic acid** (g/L)*		**Functional peptide** (mg/L)*	
	Lab	**Pilot**	**Lab**	**Pilot**	**Lab**	**Pilot**
End of fermentation (t_{24})	8 logs	9 logs	5.4 ± 0.20^{a}	6.33 ± 0.74^{b}	17.23 ± 0.28^{c}	22.22 ± 0.88^{d}
Heat treated & spray-dried	< 2 logs	< 2 logs	4.8 ± 0.30^{a}	5.20 ± 0.74^{b}	18.40 ± 0.92^{c}	21.90 ± 0.30^{d}

*Lactic acid and functional peptide concentrations reported for heat treated and spray-dried rice powders were referred to the rehydration volume (1:15 w/v), used for reproducing the solid to water ratio of the fermentation batch.

4.4.2 Comparison lab/pilot scale results for milk

As shown in Figure 4.30, the bacterial growth curve followed the same trend in both lab and pilot scales. A lag phase of 2 h, an exponential phase up to 14 h of fermentation and a following stationary phase up to the end of the process (t_{24}) were observed in both cases.

The maximum bacterial charge was achieved in both cases at time t_{20} with $1.9 \times 10^{8} \pm 1.2 \times 10^{8}$ CFU/mL at lab scale and a slightly higher bacterial load of $6.7 \times 10^{8} \pm 3.75 \times 10^{8}$ CFU/mL at pilot scale.

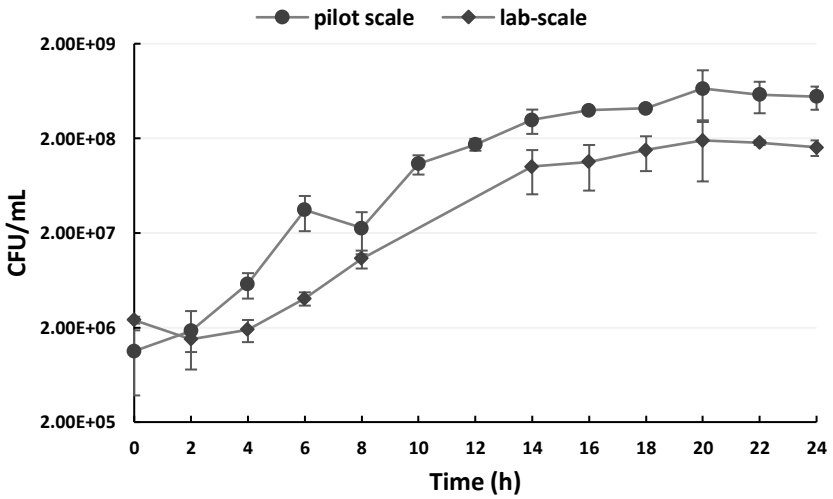

Figure 4.30. Comparison between laboratory (protocol MF2) and pilot scale milk growth curve.

Figure 4.31 and Figure 4.32 show the comparison between laboratory and pilot scale lactic acid and peptide production, respectively.

In both cases, the lactic acid production curve showed a non-growth-linked trend and the maximum production was registered after 24 h of fermentation with a higher concentration (2.1 ± 0.25 g/L) at pilot scale, compared to the lab scale value (1.2 ± 0.3 g/L).

As for the functional peptide production, the non-growth linked trend was similar in both lab and pilot scales, reaching a higher peptide content of 14.2 ± 0.8 mg/L at time t_{24} at pilot scale than that detected in fermented milk at lab scale after 24 h of fermentation (6.93 ± 0.5 mg/L).

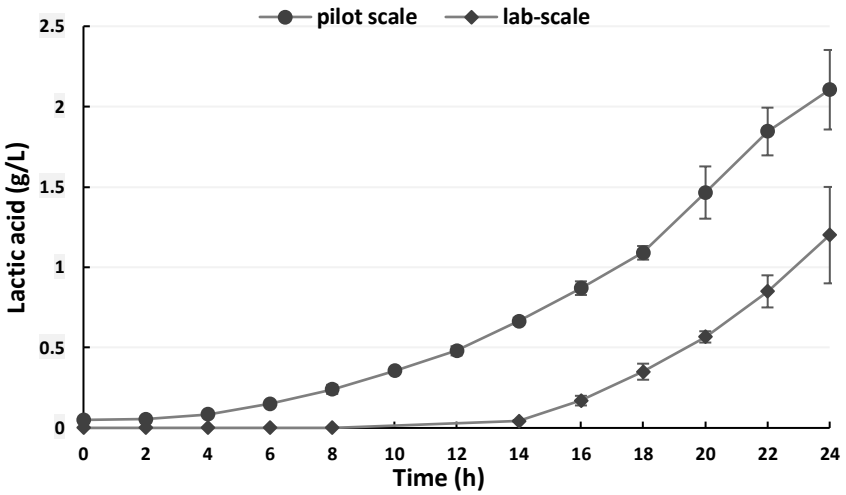

Figure 4.31. Comparison between laboratory (protocol MF2) and pilot scale milk lactic acid production curves.

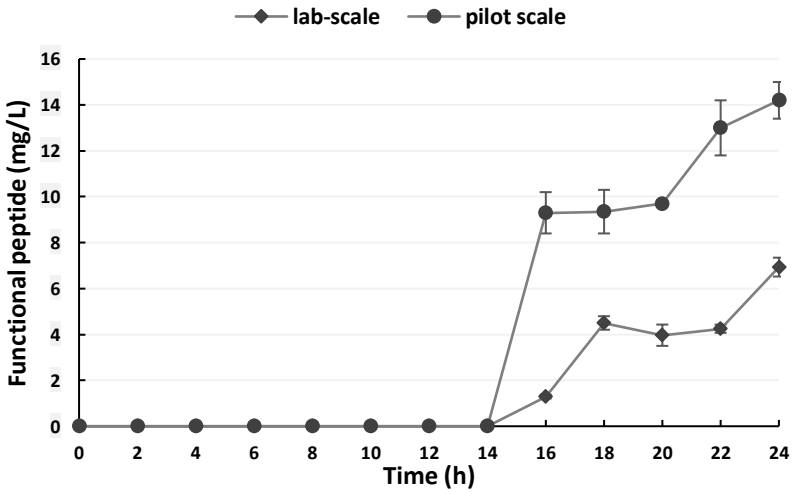

Figure 4.32. Comparison between laboratory (protocol MF2) and pilot scale milk functional peptide production curves.

Deactivation and drying

As well as for rice, the stabilizing treatments (heat and drying treatments) on fermented milk had a low impact on the quality of the product that was confirmed for both scales, as reported in Table 4.19. Statistically significant differences between lactic acid and peptide concentrations determined for lab and pilot scale trials were observed, both before and after stabilization processes.

Table 4.19. Comparison between bacterial growth, lactic acid, and functional peptide concentration in fermented milk, before and after stabilizing treatments performed at laboratory and pilot scale. The values marked with different lowercase letters in the same arrow are significantly different (t-student test provided $p < 0.05$).

Sample	**Bacterial charge** (CFU/mL)		**Lactic acid** (g/L)*		**Functional peptide** (mg/L)*	
	Lab	**Pilot**	**Lab**	**Pilot**	**Lab**	**Pilot**
End of fermentation (t_{24})	8 logs	8 logs	1.2 ± 0.30^{a}	2.1 ± 0.25^{b}	6.93 ± 0.50^{c}	14.2 ± 0.80^{d}
Heat treated & spray-dried	< 2 logs	< 2 logs	0.9 ± 0.30^{a}	1.90 ± 0.23^{b}	7.20 ± 0.20^{c}	13.9 ± 0.51^{d}

*Lactic acid and functional peptide concentrations reported for heat treated and spray-dried milk powders were referred to the rehydration volume (1:10 w/v), used for reproducing the solid to water ratio of the fermentation batch.

5 Conclusions

This PhD project allowed the development of standardized protocols, both at lab and pilot scales, to produce milk, cereal and leguminous based postbiotic functional foods.

The substrates chosen for this work, milk, rice flour and beans, provided by Kraft Heinz Company, represent the main investigation fields on which, in recent years, the scientific research on functional foods has been based: i) dairy based functional foods, considered the most common probiotic substrates for several years; ii) non-dairy based functional foods, which recently have been gaining increasing interest for their gut health enhancing effects and their ability to satisfy a wider range of consumers, even those affected by intolerances and different dietary habits.

The process consisted of three phases: fermentation, inactivation phase and the drying phase (to obtain a product stable over time). Each of these phases was tested first at laboratory scale in order to obtain adequate recipes and protocols for each feedstock to be implemented at pilot scale.

Previous studies carried out on rice and milk substrates allowed for a rapid and effective optimization of lab-scale protocols so that pilot scale experiments could be carried out.

Conversely, fermentation of a leguminous substrate had been an unexplored field until then and, for this reason, more detailed investigations at laboratory scale were needed, before the experiments could be also conducted efficiently at a pilot scale. Once the necessary pre-treatments were optimized, fermentation trials were carried out using *Lactobacillus paracasei* CBA L74 patented and provided by Kraft Heinz Company. Bacterial growth, lactic acid production and the amount of a functional peptide reputed as the functional metabolite responsible for the immunomodulatory activity of the obtained postbiotic were monitored throughout the fermentation process.

At laboratory scale:

- Fermentation of a hydrolyzed rice flour suspension using amylase, performed under pH and temperature-controlled conditions, provided an increase in the bacterial growth of 3 logs, with a maximum load of $5.3 \times 10^8 \pm 4.7 \times 10^8$ CFU/mL after 18 h of fermentation. A maximum lactic acid concentration of 5.4 ± 0.2 g/L was reached after 24 h of process and an increasing amount of the functional peptide was detected during the process, up to a maximum concentration of 17.23 ± 0.28 mg/L after 24 h of fermentation.

  A better duplication capacity of the microorganism during the exponential growth phase was observed when pH of rice medium was controlled at the optimal value of 6.2, ensuring higher generation number and constant growth rate values (8.52 and 0.71 h^{-1}, respectively) than those evaluated when no pH control was implemented.

The kinetic analysis confirmed that the sugar content in rice medium widely satisfied the nutritional requirement for the microorganism growth, without any inhibiting effect.

- Fermentation of milk performed under pH and temperature-controlled conditions, provided an increase in the bacterial growth of 3 logs, with a maximum charge of $1.9 \times 10^8 \pm 1.2 \times 10^8$ CFU/mL reached after 20 h of fermentation. Lactic acid and peptide concentrations reached their maximum values of 1.2 ± 0.3 g/L and 6.93 ± 0.5 mg/L, respectively, after 24 h of process. Both concentrations were lower than those obtained for lactic acid and functional peptide during rice flour fermentation. For milk, the implementation of the pH control allowed a slight improvement in duplication capacity of the microorganism during the exponential phase, represented by a lower doubling time of 1.87 h and a higher constant growth rate of 0.53 h^{-1} than those obtained without controlling pH (1.97 h and 0.50 h^{-1}, respectively).

  The kinetic analysis suggested that the added glucose percentage in milk recipe could be reduced, as well as in rice, where 1% of added glucose was sufficient to satisfy the microorganism's nutritional requirement. In this way, it could be possible to prevent its inhibition effect on the bacterial growth, ensuring at the same time a concentration in the medium that does not limit the growth rate.
- An aqueous suspension of cooked beans was fermented without pH control in two different reactor conditions. The mixing system was improved by designing an impeller that guarantees a more homogeneous distribution of the nutrients necessary for the proliferation of the microorganism and its metabolic activity. In particular, a faster achievement of the stationary values for microbial load and lactic acid (approximately 1×10^9 CFU/mL at time t_{14} and 2 g/L at time t_{16}) was obtained and a higher constant growth rate (0.65 h^{-1}) and a lower doubling time t_d (1.53 h) were determined, in comparison with the values obtained through the non-optimized mixing system (i.e., 0.53 h^{-1} and 1.87 h for the constant growth rate and the doubling time, respectively). The functional peptide was not found in the fermented bean suspension at the end of the process, suggesting the necessity to optimize the fermentation protocol for this purpose, implementing pH control or glucose addition, as well as for rice and milk.

  The kinetic study also suggested that the optimization of the glucose content in the medium and the pH control could be useful strategies to reach a higher specific growth rate and improve the kinetic performances of beans.
- A mild heat treatment at 90°C for 1 min, for rice and milk, and at 90°C for 20 min for beans was identified to reduce the bacterial load in the fermented substrates, without seriously impacting on the quality of the products (physical changes were not noticed and a slight

decrease in lactic acid concentration was recorded). Moreover, freeze-drying and spray-drying methods were tested to evaluate their impact on the product.

The two drying technologies presented the same impact on the products (lactic acid and peptide concentration remained constant), meaning that they can be interchangeable and, although freeze-drying is considered giving high-quality food products, spray drying can be used, saving the expenses and obtaining dried products in shorter times.

All these processes were tested on rice and milk at pilot scale, with a very efficient and totally controlled scaling-up. Fermentation results, in terms of bacterial load, lactic acid and functional peptide concentrations, were comparable to those obtained at laboratory scale for each feedstock.

As for milk, a maximum growth of $6.70 \times 10^8 \pm 2.65 \times 10^8$ CFU/mL (t_{20}), a maximum lactic acid concentration of 2.10 ± 0.20 g/L (t_{24}) and a maximum peptide production of 14.20 ± 0.80 mg/L (t_{24}) were observed, while a maximum growth of $1.27 \times 10^9 \pm 6.90 \times 10^8$ CFU/mL (t_{20}), a maximum lactic acid production of 6.30 ± 0.20 g/L (t_{24}), and a maximum peptide concentration of 22.60 ± 4.10 mg/L (t_{20}) were achieved for rice. Moreover, it was confirmed that the inactivation protocol (90°C for 1 min) was efficient to kill the bacteria in the product, also with a larger scale, and that spray drying technology had a low impact on the product quality.

Finally, a shelf-life analysis was carried out on the powders produced at pilot scale, testing three different storage temperatures (4°C, 20°C and 37°C). The rice powder turned out to be more stable over time, changing its organoleptic and chemical properties in a negligible way, after six months, whereas milk powder (especially when stored at 37°C) changed its features after three months and in an unacceptable way, in particular from an organoleptic point of view.

Future prospects include a further optimization of the process, looking at the maximization of the concentration of the functional compound identified, to provide a higher value to the product from both a functional and then a commercial point of view. Furthermore, to have a complete functional characterization, the fermented product could be characterized in terms of radical scavenging activity, total phenolic compounds, GABA, resistant starch, and serotonin concentration.

Moreover, a preliminary evaluation of the effect of the fermentation process on the reduction of antinutrient compounds contained in leguminous substrates was performed.

LP CBA L74 seems to be not able to reduce the content of indigestible oligosaccharides, such as raffinose and stachyose, responsible for gut health diseases, such as constipation, bloating and flatulence. A future purpose will be to investigate the action that this microorganism could have on other antinutrient factors, as allergenic proteins, proteinase inhibitors and phytates, which limit their consumption and affect the digestibility and bioavailability of nutrients.

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7 Appendix

A1. *Animal Free broth (AFB) preparation*

Bacto yeast extract (BD) 20 g/L, magnesium sulphate 0,5 g/L (Sigma Aldrich), dextrose 50 g/L (Sigma Aldrich), citric acid 0,5 g/L (Sigma Aldrich). Two bottles of water are prepared; one with Yeast extract, citric acid and magnesium sulphate and the other only with the dextrose.

The two bottles are subjected to a standard autoclave cycle for the sterilization. Once the cycle is finished, the content of the two bottles is mixed.

A2. *Strain reactivation protocol (Lab and pilot scale)*

For lab scale, the strain is conserved at -26°C in cryovials containing 1 ml of animal free broth with a bacterial charge of 8 log; also, glycerol (20%) is added to avoid the formation of ice crystals that could damage the cells during the freeze storage. The reactivation of the strain takes place adding 1 mL of the thawed strain to 9 ml of fresh broth, incubating it at 37 °C for 24 h.

For the pilot scale, the reactivation takes place in 48 h.

From the Working Cell bank stored at -26°C, a single glass bead is taken and added to 10 ml of fresh animal free broth and incubated for 24 h at 30 °C, obtaining the pre-inoculum.

Subsequently, the pre-inoculum is added to 2 L of fresh animal free broth and is incubated for 24 h at 30°C. In both cases, the inoculum and fermentation medium volume ratio must be 1:100.

A3. *Sieving operation on milled beans.*

In order to standardize the bean substrate to be fermented and to fix the granulometric distribution of bean flour prepared by milling dried beans provided by Kraft Heinz Company, a sieving operation using mean stainless-steel sieves was performed.

The particle size distribution of the flour ($d_{average}$ = 110 µm) used for the preliminary fermentation tests is reported in Figure 11.1.

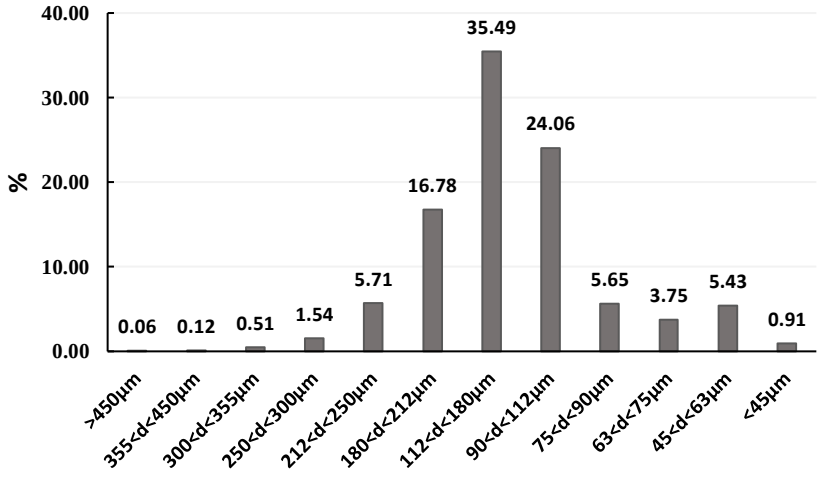

Figure 7.1 Granulometric distribution of bean flour.

A4. *Serial diluition and spread plate method*

Direct plate counting is a method used to count the number of viable cells in a sample.

There are obvious disadvantages. First, plate count method requires long incubation times. It is often hampered by technical difficulties such as clumping and inhibition by neighbouring cells. The choose of enumeration medium and incubation conditions for specific species may also be challenging.

Cells to be counted have been diluted (Figure 11.2) due to the fact that too many cells will cause the Petri plate to be so densely populated with colonies, that they would be impossible to count.

After this operation, they are incubated on an agar medium until colonies form.

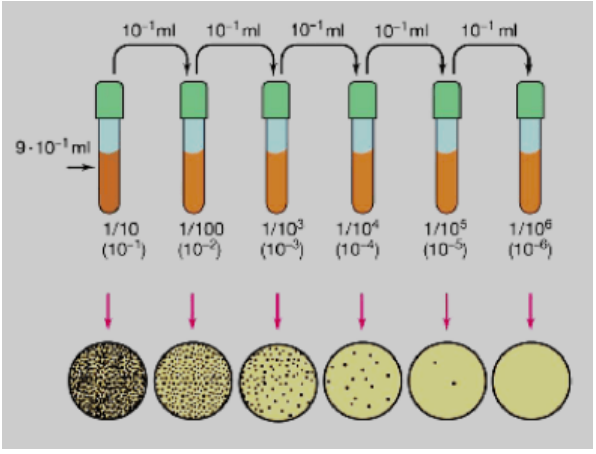

Figure 7.2. Serial diluition procedure.

There are two main methods of direct plate counting: spread plate method and pour plate method.

The spread plate method, that is used in the experimental analyses, consists of evenly spreading the diluted sample over an agar plate (Figure 11.3). Briefly, a volume of 0.1 ml of the diluted sample is

pipetted and spread over agar surface by a sterile spreader, since the agar will not be able to absorb the excess. Using this method colonies are obtained which form on the surface of the agar.

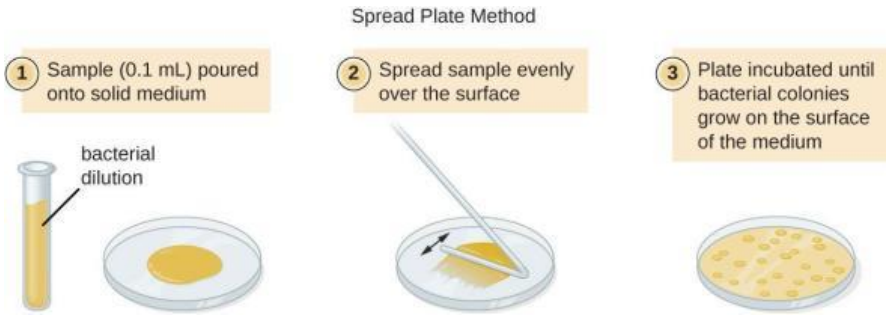

Figure 7.3. Spread plate method.

A5. *MRS agar preparation*.

Suspend 62 g in 1 L of distilled water. Boil to completely dissolve the medium and sterilise by autoclaving at 121 °C for 15 min.

A6. *Gelisate agar preparation*:

Suspend 20 g in 1 L. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121 °C for 15 min.

A7. *MacConkey agar preparation*:

Suspend 51.5 g in 1 L of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 min.

A8. *MYP agar preparation:*

Suspend 21.5 g in 450 mL distilled water and bring gently to the boil to dissolve. Sterilise by autoclaving at 121 °C for 15 min. Cool to approximately 49 °C and aseptically add 50 mL Egg Yolk Emulsion and 1 vial of Polymyxin B Supplement, reconstituted as directed. Mix well and pour into sterile petri dishes.

A9. *Pre-treatment sample for acids analysis*.

For lactic acid analysis, it is necessary to weight 2 g of sample and to add 1 mL of Carrez I, 1 mL of Carrez II and 2 mL of NaOH 0.1 M, then to bring the solution to 50 mL with distilled water.

For the elaboration of the results a x25 dilution has to be taken into consideration.

For butyric, acetic and propionic acid analysis, the same samples pre-treated as above are used, adding esanoic standard 5 g/L in a 1:1 ratio.

Publications

Publications linked to PhD activity:

- Colucci Cante, R., Gallo, M., Nigro, F., Passannanti, F., Budelli, A., Nigro, R. (2021). Mathematical modeling of Lactobacillus paracasei CBA L74 growth during rice flour fermentation performed with and without pH control. *Appl. Sci.*, 11, 2921. DOI: 10.3390/app11072921

- Colucci Cante, R., Gallo, M., Nigro, F., Passannanti, F., Salameh, D., Budelli, A., Nigro, R. (2020). Lactic fermentation of cooked navy beans by Lactobacillus paracasei CBA L74 aimed at a potential production of functional legume-based foods. *Can. J. Chem. Eng*., 98 (9), 1–7. DOI: 10.1002/cjce.23817

- Salameh, D., Nigro, F., Colucci Cante, R., Passannanti, F., Gallo, M., Budelli, A., Marzocchella, A., Nigro, R. (2019). Fermentation of Rice Flour Supernatant using Lactobacillus Paracasei CBA L74. *Chem Eng. Trans.*, 75, 289-294. DOI:10.3303/CET1975049

- Gallo, M., Passannanti, F., Colucci Cante, R., Nigro, F., Salameh, D., Schiattarella, P., Schioppa, C., Budelli, A., Nigro, R. (2019). Effects of the glucose addition during lactic fermentation of rice, oat and wheat flours. *Appl. Food Biotechnol.,* 7 (1), 21–30. DOI: 10.22037/afb. v7i1.26975

- Gallo, M., Passannanti, F., Colucci Cante, R., Nigro, F., Schiattarella, P., Zappulla, S., Budelli, A., Nigro, R. (2020) Lactic fermentation of cereals aqueous mixture of oat and rice flours with and without glucose addition. *Heliyon*, 6 (9): e04920. DOI: 10.1016/j.heliyon. 2020.e04920

- Gallo, M., Passannanti, F., Schioppa, C., Montella, S., Colucci Cante, R., Nigro F., Budelli, A., Nigro, R. (2021). Enzymatic pre-treatment and lactic fermentation of wheat flour suspension at a high solid content. *J. Food Process. Preserv.*, e15299. DOI: 10.1111/jfpp.15299

- Gallo, M., Passannanti, F., Schiattarella, P., Esposito, A., Colucci Cante, R., Nigro F., Budelli, A., Nigro, R. (2021). Banana Puree Lactic Fermentation: The Role of Ripeness, Heat Treatment, and Ascorbic Acid. *Appl. Sci.*, Under Review.

Publications linked to different research activities:

- Colucci Cante, R., Prisco, I., Garella, I., Gallo, M., Nigro, R. (2020). Extracting the lipid fraction from waste bilberry seeds with a hydrofluorocarbon solvent. *Chem. Eng. Res. Des*., 157, 174-181. DOI: 10.1016/j.cherd.2020.02.032
- Colucci Cante, R., Garella, I., Gallo, M., Nigro, R. (2021). Effect of moisture content on the extraction rate of coffee oil from spent coffee grounds using Norflurane as solvent, *Chem. Eng. Res. Des*., 165, 172-179. DOI: 10.1016/j.cherd.2020.11.002

Conference Proceedings

- Presentation at Mediterranean Combustion Symposium, Tenerife, Spain, 16-20 June 2019, Feasibility as liquid fuel of lipid fractions from spent coffee for spray combustion, Colucci Cante, R., Garella. I., Allouis, C., Cimino, S., Stanzione, F., Nigro, R.

- Poster for Conference GRICU 2019, Mondello, "Lactic Fermentation of a Navy Beans by Lactobacillus paracasei CBA L74 aimed at a potential production of functional legume-based foods", Colucci Cante R., Gallo M., Nigro F., Passannanti F., Salameh D., Budelli A., Nigro R.