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Characterization of lactic acid bacteria from a kvass-like beverage produced using buckwheat (*Fagopyrum esculentum*) flour

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Student:

ANGELICA COMPAGNONI

Supervisor:

PROF. ANDREA OSIMANI

Assistant supervisors:

PROF. JOANNA HARASYM

DR. GIORGIA RAMPANTI

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ACRONYMS AND ABBREVIATIONS

ABV   Alcohol by volume

CFU   Colony forming unit

DNA   Deoxyribonucleic Acid

EDTA   Ethylenediaminetetraacetic Acid

EPS   Exopolysaccharides

FB   Fresh bread

FOSs   Fructo-oligosaccharides

GOSs   galacto-oligosaccharides

HePS   Heteropolysaccharides

HoPS   Homopolysaccharides

IK   Italian buckwheat kvass-like beverage

LAB   Lactic acid bacteria

MRS   Man, Rogosa e Sharpe

PCR   Polymerase Chain Reaction

PCR-DGGE   Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis

PK   Polish buckwheat kvass-like beverage

qPCR   Quantitative Polymerase Chain Reaction

rRNA   Ribosomal ribonucleic Acid

SB   Stale bread

SBBE   Sterile buckwheat bread extract

INTRODUCTION AND AIM OF THE THESIS

In the contemporary landscape of the food industry, there is a growing interest in innovation and sustainability. In this context, the valorization of food waste and the use of traditional techniques for the development of new products play a key role.

This thesis explores the development of an innovative beverage using stale buckwheat bread as a raw material. The process focuses on the development of two variants of this beverage, one derived from Italian buckwheat and the other from Polish buckwheat, following a traditional Eastern European recipe known as kvass.

Buckwheat is an ancient crop renowned for its nutritional qualities, with one of its most noteworthy attributes being its gluten-free composition. This characteristic makes it an ideal dietary option for individuals with coeliac disease, as it can be consumed without triggering adverse gluten-related reactions. Moreover, using stale buckwheat bread to produce kvass not only reduces food waste but also allows for the exploration of spontaneous fermentation and the acquisition of knowledge about the production of buckwheat-based drinks.

The production of kvass-like beverages involves a homemade process that includes staling and drying the bread, followed by boiling and filtering the mash. Subsequently, a 24h-dough, prepared using the same bread flour, is introduced into the mash, mixed thoroughly, and left to ferment. Kvass-like beverages were assessed through physico-chemical (pH, solid matter, color) and microbiological analyses (viable counting) for five days after bottling. Moreover, a total of 36 lactic acid bacteria were isolated from the beverages, identified, and characterized in order to select potential starter cultures for kvass production.

This thesis aims to advance scientific understanding in the field of functional and sustainable beverage production. A fundamental aspect is assessing whether traditional home production methods can effectively integrate this raw material, testing the fermentation process, and providing detailed information on the microbial environment involved. This will aid in developing starter cultures suitable to standardized commercial production, thereby valorizing a product that would otherwise be considered a food waste, thus enhancing the sustainability of the production process.

CHAPTER 1

PROPERTIES AND USES OF BUCKWHEAT FLOUR: DEVELOPMENT OF FUNCTIONAL FERMENTED CEREAL-BASED BEVERAGES

1.1 Morphological and physiological characteristics of buckwheat

Buckwheat (*Fagopyrum esculentum* Moench), a pseudocereal belonging to the Polygonaceae family, is an alternative crop usually grouped with cereals due to similarities in cultivation and utilization, although it is not a true cereal grain. Cereals are the caryopses (fruit and seed in a single structure) of herbaceous monocotyledonous plants, known as Gramineae, while pseudocereals are the seeds of herbaceous dicotyledonous plants from distinct families (Unal et al., 2017).

The buckwheat seed, known as achene, has a hard outer hull that surrounds the seed coat, endosperm, and embryo. The embryo is located in the center of the endosperm, which contains large amounts of proteins, starch, and vitamins (Pomeranz et al, 1983). Both cereals and buckwheat have starchy endosperms and a non-starchy aleurone layer, making them similar in some respects. Despite other parts of the buckwheat plant being usable for human consumption and animal feed, buckwheat is primarily grown for seed production (Biacs et al 2002; Mazza and Oomah 2005).

The buckwheat plant has a taproot system, an erect and cylindrical stem that can reach heights of 60-120 cm, lanceolate or cordate-triangular leaves, and raceme inflorescence with white or pink hermaphrodite flowers. The fruit is a silvery-brown or greyish trigonal achene. Although the root system is not extensive, its physiological activity is significant; the roots excrete formic, acetic, citric, and oxalic acids to help absorb nutrients, mainly phosphorus, from less accessible forms. Harvesting occurs in September or October, depending on the variety and seasonal conditions (Björkman, 2003).

There are two major species of buckwheat: Common buckwheat (*F. esculentum* Moench) and Tartary buckwheat (*F. tataricum* Gaertner). Common buckwheat is the most widely grown

species (Marshall and Pomeranz, 1981; Mazza and Oomah, 2005), native to south-central China and Tibet, and has been introduced to suitable climates across Eurasia, Africa, and the Americas. Tartary buckwheat is mainly grown in Asia. Buckwheat can be planted as a warm-season forage crop or as a standalone crop, requiring minimal seedbed preparation. It is known for its rapid growth, maturing within seven to ten-weeks (Tahir and Farooq, 1988; Tsuneo, 2004). It thrives in various environmental conditions, including marginal lands and rocky, poorly tilled soils, and can improve soil fertility with low fertilization needs. Sowing typically occurs from late May and June-July, with rows spaced 15-20 cm apart (Alencar et al., 2019). Buckwheat is often included in the category of ancient grains, which refers to grains first domesticated thousands of years ago (approximately 8,000 years ago in the case of buckwheat, likely in southwest Asia) and then grown only as a minor food in the modern era before being "rediscovered". Its cultivation has declined over the last century, primarily due to self-incompatibility and breeding difficulties. (Myers, 2018).

Buckwheat offers numerous agronomic benefits, including high nutrient use efficiency, low fertilizer requirement, and strong competitiveness against weeds (Jung et al., 2015; Siracusa et al., 2017), making it a valuable component of sustainable cropping systems (Joshi et al., 2019). It also enhances the presence of important pollinator species in agriculture (Taki et al., 2009), supporting low-input and organic production systems. Commercial beehives are often placed adjacent to buckwheat crops to increase pollination and seed set (Kasajima et al., 2017), and the resulting buckwheat honey has proven therapeutic properties (Schramm et al., 2003). Additionally, buckwheat is commonly used in cover crops mixtures due to its rapid growth, soil coverage, weed suppression, and ease of management (Brust et al., 2014; Saunders Bulan et al., 2015).

1.2 World buckwheat market

Buckwheat is currently cultivated in a vast area, including large regions of Asia (India and other countries along the Himalayas, China, Korea, Japan, former Soviet Central Asia), and Europe (Russia, Belarus, Ukraine, Poland, the Baltic States, France, and to a lesser extent other countries in Central and Eastern Europe and along the Alpine chain) (Tallarico et al., 2014). Global buckwheat production reached 3,827,748 tons across 3,940,526 hectares (Faostat, 2017). Over the last 40 years, China has been the largest producer of buckwheat, but currently Russia leads with 1,524,280 tons produced on 1,497,783 hectares. In Poland, buckwheat is cultivated on approximately 114,000 hectares, representing 1.6% of the total sowing structure and slightly more than 1% of the cereal sowing structure (FAOSTAT, 2022). Untill the 1980s,

buckwheat production in Poland was less than 1 ton per hectare, limiting the scale of production. However, in the last decade, there has been a clear improvement in the yield of this pseudocereal (Kwiatkowski, 2023).

Historically, buckwheat, along with rye, was a dietary staple before the introduction of improved cold-resistant wheat varieties (Tallarico et al., 2014). The largest importer of buckwheat is Japan (70,265 tons in 2010), followed by Papua New Guinea (41,000 tons) and Italy (19,691 tons). Italy has a substantial deficit, importing more than 90% of tis consumption needs, primarily from China, with minimal exports (156 tons in 2010) (FAOSTAT, 2013). Buckwheat cultivation was introduced in Italy around the 16th century (Fontanari, 2004). However, cultivation has significantly declined in recent decades, now surviving only in a few Alpine valleys (Borghi et al., 1995; Borghi, 1996), where the flour is used in many traditional recipes.

The increasing demand for buckwheat in Italy could be met by reintroducing the crop in suitable soil and climate environments, particularly in the hilly and mountainous areas of Central and Southern Italy. Moreover, numerous studies have highlighted buckwheat's superior nutraceutical qualities compared to other cultivated cereals (Tallarico et al., 2014). This opens the way for adopting buckwheat cultivation as an alternative and innovative crop in these regions.

In summary, buckwheat cultivation in Italy could become a promising investment due to its potential role in future sustainable cropping systems. Meanwhile, in Poland, where buckwheat is already widely cultivated, exploring food product development using this raw material could yield significant prospects.

1.3 Buckwheat flour: beneficial properties and use in bread-making

Dehulled buckwheat seeds, known as raw groats (Marshall & Pomeranz 1982; Ikeda 2002), are primarily used for human consumption as breakfast cereals or processed into flour for various bakery products such as bread, cookies, snacks, and noodles.

Buckwheat seeds contain 59% to 69% starch, composed of 15-25% amylose, 7-37% resistant starch, and amylopectin (Skrabanja et al., 2004; Christa et al., 2008). Starch is the dominant component influencing the functional properties of flour-based food; achieving suitable textural properties for buckwheat-based pasta and other products requires a balanced ratio of protein to starch (Ikeda et al., 1997).

Buckwheat flour is darker and rougher than durum wheat flour and has a rustic and intense flavor. It contains numerous nutraceutical compounds (Li & Zhang 2001) and is rich in

vitamins, mainly those of B group (Fabjan et al. 2003). The amino acid composition of buckwheat proteins is well-balanced and with a high biological value (Kato et al. 2001), although the protein digestibility is relatively low (Liu et al. 2001).

Consumption of buckwheat and buckwheat-enriched products is associated with various health benefits, including hypocholesterolemic, hypoglycemic, anticancer, and anti-inflammatory effects (Lukšič et al., 2016). Buckwheat grains are a significant source of microelements, such as Zn, Cu, Mn, Se (Stibilj et al. 2004), and macroelements like K, Na, Ca, Mg (Wei et al. 2003). With 80% unsaturated fatty acids, over 40% are polyunsaturated fatty acid (PUFA) (Krkošková & Mrázová 2005). The significant contents of rutin, catechins, and other polyphenols enhance dietary value of buckwheat exerting antioxidant activity (Oomah & Mazza 1996; Wanatabe 1998). Buckwheat bran and hulls exhibit 2–7 times higher antioxidant activity than barley, triticale, and oats (Holasova et al., 2002). Buckwheat grains are also rich in dietary fiber and resistant starch, useful in preventing obesity, diabetes, and colon cancer (Brennan, 2005; Lu et al., 2015).

In the food industry, the trend towards functional foods has made pseudocereals like buckwheat popular due to their exceptional nutritional and nutraceutical value, as well as being gluten-free. These properties have earned pseudocereals the label "the grains of the twenty-first century" (FAO, 2011). Buckwheat flour is suitable for gluten-free diets, a growing market due to increasing awareness and diagnosis of gluten intolerance (Miranda et al, 2014). Gluten-free products enriched with buckwheat flour offer health-promoting components such as fiber, antioxidants, and minerals, though the modification of traditional production processes might be necessary (Burluc et al, 2012). Gluten-free bread incorporating buckwheat flour improves technological quality and enriches the final product with proteins and microelements like Cu and Mg (Krupa-Kozak et al., 2011). However, the sensory and baking quality of gluten-free products can be a challenge due to the unique viscoelastic properties of gluten (Saturni et al., 2010; Hager et al., 2012). Efforts to improve gluten-free bread's functional and sensory quality have shown promise, with studies indicating higher volume and softer crumb in buckwheat-based gluten-free bread compared to controls (Álvarez-Jubete et al., 2010; Torbica et al., 2010). Despite these advancements, more research is needed to develop high-quality, low-glycemic index gluten-free buckwheat bread (Wolter et al., 2013).

1.4 Fermented cereal-based beverages

Fermentation is one of the oldest known food processing methods, with its history stretching back to the Neolithic period (Tsafrakidou et al, 2020). Archaeological findings suggest that

over 9000 years ago, individuals were already fermenting beverages (McGovern et al, 2004). Remnants in jars and vessels indicate that winemaking was popular in Neolithic Egypt and the Middle East (Prapajati et al, 2008). Moreover, the domestication of crops, essential for beer making, marked a significant step in human evolution, as turning grains into staples required exceptional technical and culinary skills (Harmon, 2009).

Fermentation can be categorized based on primary metabolites produced: (a) alcoholic fermentation, conducted by yeasts, producing ethanol and CO_2 as primary products; (b) acetic fermentation, conducted by *Acetobacter* spp., producing acetic acid as primary product; (c) lactic fermentation, involving lactic acid bacteria (LAB), producing lactic acid as main metabolic product; (d) ammonia or alkali fermentation of proteinaceous substrates by various species of bacilli and fungi (Marco et al, 2017).

Fermentation technology has gained renewed interest for its role in developing safe products with unique nutritional and functional attributes (Tsafrakidou, 2020). Despite some drawbacks as raw materials, such as deficiency in certain amino acids, lower protein content, and the presence of antinutrient compounds compared to animal or dairy foods, fermented cereals are nutritionally superior to their native counterparts (Blandino et al, 2003). As plant-based matrices, fermented cereal products are suitable for people with lactose intolerance, milk allergies, or those following low lipid or vegan diets.

In the last decade, the rise in food intolerances, allergies, and food-based lifestyle choices has increased consumer demand for functional foods designed for a healthy and balanced diet. Cereal-based beverages are part of this category due to their health benefits (Coda et al., 2017). Fermentation enhances the palatability of the grains by improving texture and flavor, reducing the need for additional flavorings or additives (Ignat et al., 2020).

Originally, cereal-fermented beverages were produced for preservation purposes. Some of these beverages, now commercially available as soft drinks and non-alcoholic beverages, were traditionally prepared as alcoholic beverages (Marsh et al., 2014). According to EU regulation no. 1169/2011, a beverage must be labeled as an alcoholic drink if its alcoholic strength by volume (Alcohol By Volume, ABV) exceeds 1.2%.

Globally, there are numerous non-alcoholic cereal fermented beverages with similar profiles, targeting thirst-quenching properties, nutrition value, and cultural significance. Fermented cereal beverages are common around the world, especially in developing countries, and are generally made using spontaneous microbial cultures (Marsh et al.,2014). Some examples of non-alcoholic cereal-based fermented beverages are listed in Table 1 (Ignat et al., 2020).

Table 1: Non-alcoholic fermented cereal-based beverages (Ignat et al., 2020).

Beverage	Sensory attributes	pH	Production scale	References
Amazake	Cloudy appearance, sour-sweet taste	3.9	Homemade and industrialised	Ajiro et al. (2017); Kawakami, et al. (2020); Oguro ewt al. (2017)
Bors	Sour-bitter taste; odour notes: "bran", "yogurt", "pungent/sour"	3.3-4.2	Homemade and industrialised	Nicolau et al. (2016); Grosu-Tudor et al. (2019)
Boza	Thick liquid, pale yellow coloring; sweet-sour taste	2.93-5.3	Homemade and industrialised	Kedia et al. (2007); Ramakrishnan et al. (2019); Akpinar-Bayizit et al. (2010)
Busa	Thick homogeneous suspension, light to dark beige; sweet-sour taste	3.4-5.3	Homemade;	Gotcheva et al. (2001); Ramashia et al. (2019)
Gowé	Brown/white color; sweet, acidic, cereal taste; soft texture	3.5-4.7	Traditional, small-scale processors	Adinsi et al. (2015); Adinsi et al. (2017)
Kunun-zaki	Low viscosity, creamy appearance; sweet-sour taste	3.8	homemade, local producers	Agarry et al. (2010); Nkama et al. (2010)
Kvass	Slightly cloudy appearance, light-dark brown color; sweet-sour taste	3.2-4.3	traditionally homemade; industrialised differently than the traditional approach	Basinskiene et al. (2016); Gambus et al. (2015)
Mahewu/ Amahewu	Creamy color, sour taste	3.5	homemade, commercially produced in African countries	Olusanya et al. (2020); Idowu et al. (2016); Fadahunsi et al. (2017)
Munkoyo	Slight yellow colour; sweet, mildly sour taste	3.3-4.2	Homemade	Phiri et al. (2019); Foma et al. (2012); Chileshe et al. (2020)

Obushera	Moderately thick composition, pale brown colour; sweet and sour taste	4.5	Homemade; commercially relevant types: Obutoko, Obuteire, Ekitiribita	Mukisa et al. (2010); Byakika et al. (2019)
Oshikundu/ Ontaku	White colour, milky appearance, sweet taste	3.3-3.7	homemade; local producers	Embashu et al. (2019); Misihairabgwi et al. (2018)
Pozol	Yellow-brown colour; sweet-sour; slightly acidic taste	3.8	homemade in rural and urban areas of southeast Mexico; small	Jean-Pierre et al. (2003); Todorov et al. (2015)
Shalgam	Red colour; sour taste	3.9	home-scale level; small scale procedures	Altay et al. (2013); Coskun et al. (2017)
Tobwa/ Togwa	Opaque and brownish colour; sweet, occasionally sour taste	4	industrially produced in Tanzania	Gadaga et al. (1999); Oi et al. (2003)

1.4.1 *Kvass*

Kvass is a non-alcoholic beverage produced by fermenting kvass mash with yeast (Lidums et al., 2014). Originating from rye and barley malt, rye flour, and stale rye bread, it holds a significant cultural status in Eastern European countries, particularly in Russia, Eastern Poland, Estonia, and Lithuania. In Poland, it is commonly known as 'Kwas Chlebowy', believed to have arrived from the east around the 10th century and has remained popular for much of its history. Kvass was widely consumed between the wars after a decline during the period of partition but faced competition from Coca-Cola during the communist era.

Traditionally, spontaneously fermented kvass is made by soaking dried rye bread in hot water for several hours. The resulting water-bread extract is then fermented by adding bread yeast (Lidums et al., 2014). Two main techniques are used: one involving stale sourdough bread where all sugars needed for fermentation come from the bread itself, and the other using malt enzymes to cleave gelatinized starch (Dlusskaya, 2008).

Kvass typically has a golden-brown color, a pleasant flavor of rye bread, low sweetness, slight carbonation, and an ethanol content generally below 1%. It contains carbohydrates, proteins, amino acids, lactic and acetic acids, and vitamins derived from its raw materials or produced

during fermentation. Unlike other fermented cereal products, kvass is not heat processed post-fermentation, allowing for higher concentrations of viable yeast and lactic acid bacteria. Traditional kvass fermentations often use artisanal starters perpetuated through back-slopping, a method still widely practiced (Dlusskaya, 2008).

Most commercially available kvass beverages today are actually kvass drinks or malt extract drinks made by diluting grain extract concentrates with water, adding colorings, flavors, and artificial sweeteners (Klosse, 2013).

The production process of fermented kvass requires strict monitoring of the fermentation to ensure ethanol levels remain below regulatory thresholds and to maintain hygiene standards, thereby preventing contamination by undesirable microflora that could compromise product quality (Wong et al., 2005).

Regulation No 926/2010 establishes *"Quality and classification requirements for kvass and kvass (malt) beverage"* in the Republic of Latvia, reflecting efforts to maintain product integrity. Despite its artisanal and household-level production predominance, kvass may serve as a noteworthy example among non-alcoholic cereal-based beverages (Dlusskaya, 2008).

1.4.2 *Microorganisms involved in kvass fermentation*

The microorganisms involved in kvass fermentation typically includes lactic acid bacteria and *Saccharomyces cerevisiae* (Ermakov et al., 1982), though the specific species composition can vary significantly depending on fermentation techniques and raw materials. To date, microbial associations in kvass fermentations have been poorly investigated.

A study conducted by Dlusskaya et al. (2008) analyzed the microflora of kvass made with rye stale bread. The kvass was characterized by a combination of lactic fermentation with alcoholic fermentation. Sequence analysis of 16S rRNA identified two species of lactic acid bacteria: *Leuconostoc mesenteroides* and *Lacticaseibacillus casei*. PCR-DGGE analysis confirmed the presence of all bacterial species retrieved through cultivation, with *S. cerevisiae* dominating among the yeasts, originating from the use of baker's yeast as an inoculum.

According to Cardinale et al. (2021), buckwheat has proven to be an excellent substrate for supporting the growth of multiple strain starter cultures, including strains *L. casei* BZ21, *L. casei* BZ35, and *Lacticaseibacillus paracasei* BZ22, isolated from commercial Bulgarian boza. However, the production process described was limited to laboratory-scale production of the fermented beverage.

1.5 Bread waste valorization

In both ancient and modern times, bread remains the most consumed product globally (Sarlee, 2015). A survey conducted in 2017–2018 revealed that 40% of respondents discarded bread and pastries because they had spoiled, while 20% cited purchasing too much bread as the reason (van Gelder, 2020a, 2020b). The primary cause of substantial bakery waste is the rapid spoilage of these products. Despite supermarkets' efforts to offer discounts on bread nearing its expiration date, staling remains the main reason for bread's short shelf life (Dymchenko et al., 2023).

Bread, one of the most wasted food items, has potential as a renewable resource. A widely discussed strategy for recycling bread waste is fermentation. Bread waste contains starch, proteins, and nutrients suitable for fermentation processes (Mihajlovski et al., 2020). As bread ages, its crumb firmness increases, crust crispness decreases, and the loaf loses its fragrance, acquiring a stale taste (Hebeda & Zobel, 1996). This aging process is attributed to reduced mobility of the bread matrix caused by both recrystallizing amylopectin and loss of water from the crumb (Curti et al., 2011; Ronda et al., 2011).Starch retrogradation starts with the recrystallization of amylose and then continues with the reassociation of amylopectin, which can take several weeks (Derde et al., 2014; Monteau et al., 2017).

Bread waste is a good feedstock for microorganisms such as bacteria, molds, and yeasts. Enzymes produced by the microorganisms facilitate the saccharification and hydrolysis of polysaccharides, releasing simple sugars that can be converted into valuable products (Dymchenko et al., 2023).

Researchers have proposed several options for recycling bakery waste. According to Brancoli et al. (2020), producing feedstuffs, beer, and ethanol are among the best alternatives for recycling bakery waste. Conversely, anaerobic digestion and incineration are the least favorable options. Preventing the production of surplus bread can avoid emissions of -0.66 kg of CO_2 equivalent per 1 kg of bread, thereby contributing to global warming mitigation.

The growing global population and increasing food production and waste highlight the shortcomings of the linear economy, prompting greater interest in the circular economy. This concept focuses on integrating food waste into biological cycles through new technologies (Vea et al., 2018). Recycling food waste positively impacts environmental sustainability, and using bread waste as a raw material for new productions can lower product prices, making bread waste recycling a promising business venture (Dymchenko et al., 2023).

CHAPTER 2
MATERIALS AND METHODS

In this study, two processing lines were operated simultaneously, using two types of raw materials: Italian buckwheat flour and Polish buckwheat flour. Both variants were used to prepare bread, following identical recipes and production techniques. The bread samples were then allowed to stale for one week. The following step of the study involved the production of beverages using a traditional homemade recipe. The two kvass-like beverages produced from Italian and Polish buckwheat flours were assessed by physico-chemical and microbiological analysis. Moreover, a total of 36 lactic acid bacteria were isolated from the spontaneously fermented beverages and characterized for pro-technological and safety traits.

2.1 Bread preparation

The flours used in the production of the two types of bread were "Eko Hillar" buckwheat flour and "Sotto le stelle" buckwheat flour, from Polish and Italian market, respectively. Their nutritional tables and labels are presented in Table 2 and Figure 1, respectively.

Table 2. Nutritional tables of Polish and Italian buckwheat flours.

	Polish buckwheat flour	Italian buckwheat flour
Energy value	331 kcal	362 kcal
Fat	0,70 g	1,70 g
Including saturated fatty acids	0,01 g	0,30 g
Carbohydrates	72,00 g	75,90 g
Including sugars	2,60 g	0,10 g
Fiber	4,00 g	4,00 g
Protein	7,20 g	9,30 g
Salt	0,00 g	0,01 g
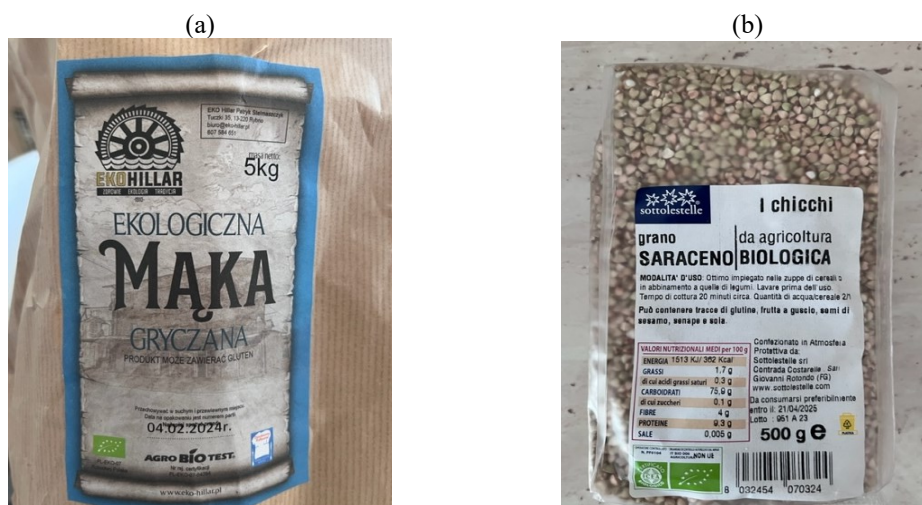
Figure 1: (a)Polish buckwheat flour label; (b) Italian buckwheat flour label.

For bread preparation, a simple recipe was used, including water, buckwheat flour, salt, and a 24h-dough (fermentation starter). This process included two main steps: the preparation of the 24h-dough (table 3) and the subsequent bread preparation (Figure 2). The procedure was replicated for both types of bread, one made entirely with Polish buckwheat flour and the other with Italian buckwheat flour.

The 24h-dough was prepared in accordance with traditional methods, mixing flour and water, followed by spontaneous fermentation for 24 hours at 25°C. The bread dough preparation involved the following ingredients: 452.5 ml of water, 440 g of buckwheat flour, 150 g of 24-dough, and 7.5 g of salt. The recipe was calculated based on 500 g of flour, in order to achieve a dough yield of 210%. The mixture was thoroughly mixed and allowed to ferment at 25 °C for 1 hour. After fermentation, the dough was baked at 240 °C for 20 minutes and then left to cool for an additional hour to ensure proper settling of the bread. After 7 days, the stale bread was sliced into small pieces and placed and dried for 72 hours at 50 °C.

Table 3: 24h-dough recipe and process parameters.

Required 24h-dough yield (%)	Buckwheat flour (g)	Water (g)	Fermentation temperature (°C)	Fermentation time (h)
250	100	150	25	24

Figure 2. Flow-chart of bread-making.

Figures 3 and 4 respectively show bread samples after cooling (fresh bread, FB, Figure 3) and after staling (stale bread, SB, Figure 4).
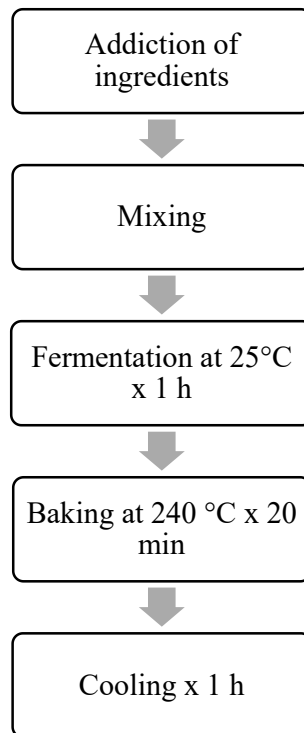
(a)   (b)
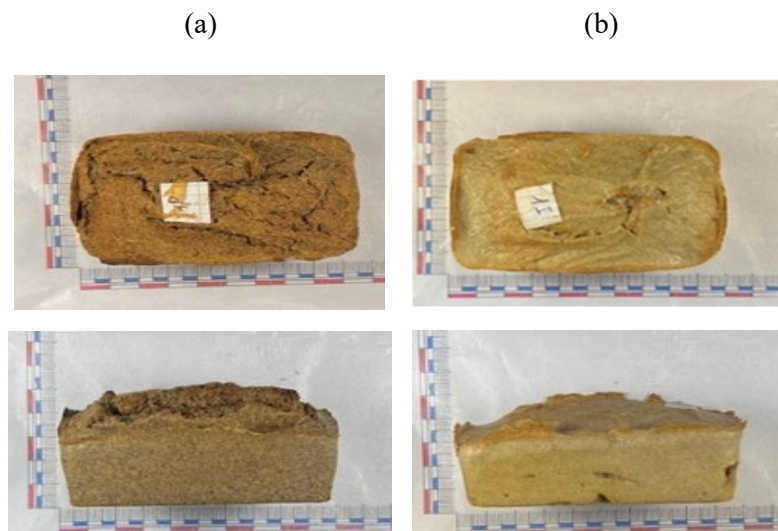
Figure 3: Fresh bread (FB). (a) Polish-buckwheat bread; (b) Italian-buckwheat bread.
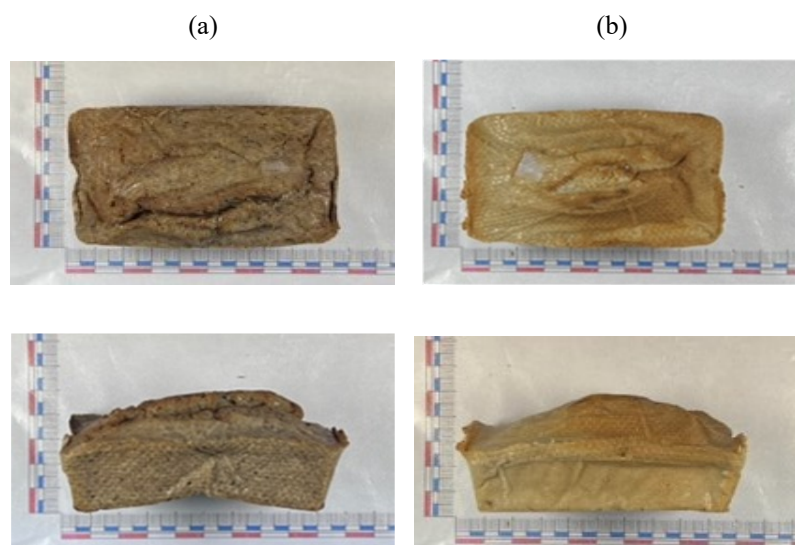
Figure 4: Stale bread (SB). (a) Polish-buckwheat bread; (b) Italian-buckwheat bread.

2.2 Kvass preparation

For the preparation of the fermented beverages, seven-day stale bread was sliced, chopped, and dried at 50 °C for 72 h. At this stage, bread was ready for mash preparation. Initially, a small-scale test was conducted by combining 100 g of dried bread with 500 ml of hot water to refine the process. For the second batch, a revised proportion was adopted, which was the basis for the analyses. The procedure followed for kvass preparation is outlined in Figure 5.

Briefly, 4 liters of water were added to 200 g of dry bread. The mixture was boiled while being stirred to form a mash. After an hour, it was strained, and 200 g of 24-hour dough was introduced (5% of the final volume). Once homogenized, the mash was transferred into 100 ml-flasks (Figure 6) and left to ferment for five days at 25°C. .

2.3 Physico-chemical analyses

The pH was measured using a pH meter equipped with a HI2031 solid electrode. The probe was inserted into the liquid sample until stabilization.

Moisture content (%) was determined by drying an aliquot of the liquid sample at 160 °C in an air atmosphere using an ATS60 moisture analyzer until a constant mass was achieved. The percentage of moisture was calculated from the weight loss of the test portion.
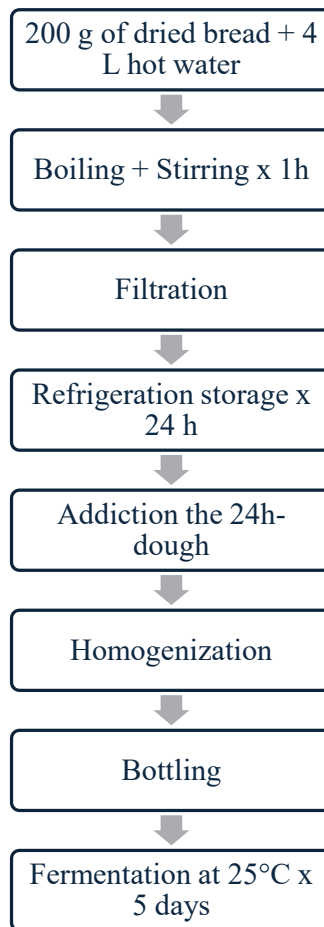
Figure 5. Flow-chart of kvass-like fermented beverages.

Figure 6: Bottled mash prior to fermentation. Left flask: Polish sample; Right flask: Italian sample.

Color measurements of kvass-like beverage samples were performed using a colorimeter with a D65 illuminant. Color was determined according to CIE L*a*b* system, where L* indicates lightness, and a* and b* are the redness/greenness and blueness/yellowness coordinates, respectively.

For each of the parameters listed above, analyses were performed in triplicate at each sampling time (from day 0 until day 5). The results were expressed as mean ± standard deviation.

2.4 Viable counts

Viable counts were determined at each sampling time (t_0, t_1, t_2, t_3, t_4, t_5,). Sterile peptone water (bacteriological peptone, 1 g L^{-1}, Oxoid, Basingstoke, UK) was used to prepare serial ten-fold dilutions from the Polish and Italian kvass-like samples. Aliquots of decimal dilutions were inoculated in duplicate on appropriate growth media for the enumeration of the following microbial groups: (i) presumptive mesophilic lactococci on M17 agar (Liofilchem, Roseto degli Abruzzi, Italy) incubated at 30°C for 48 hours; (ii) presumptive lactobacilli on de Man, Rogosa, and Sharpe (MRS) agar (VWR) incubated at 30°C for 48 hours; (iii) yeasts and molds on Rose Bengal (RB) agar (VWR) incubated at 25°C for 72 hours.

The results were expressed as the log of Colony-Forming Units (CFU) per ml of each sample and reported as mean ± standard deviation.

2.5 Isolation and characterization of Lactic Acid Bacteria

2.5.1 Isolation and identification

Colonies grown on the agar plates used for viable counting of lactic acid bacteria were randomly selected, and sub-cultured to purity on the same media used for enumeration. In order to ensure the proper conservation of the purified isolates, a solution containing glycerol as cryoprotective was utilized for each strain. In more detail, a sterile solution containing glycerol and water was mixed (1:1) with MRS broth and M17 broth for presumptive lactobacilli and presumptive lactococci, respectively. The resulting solutions were poured in the corresponding pure sub-culture plates and an L-shaped sterile spreader was used to suspend the viable biomass for each isolate. Finally, the resulting suspensions were aliquoted in sterile Eppendorf tubes, and then stored at -80°C until use.

A total of 36 isolates were obtained from the final products, 18 from the Polish buckwheat beverage and 18 from the Italian buckwheat beverage.

Total genomic DNA was extracted from the collected isolates according to Osimani et al., (2015). Briefly, a sterile loop was used to aliquot and suspend viable biomass from each pure sub-culture in an Eppendorf tube containing 300 µL of TE buffer. The resulting solutions were then treated in a Thermoblock FALC 1352 (Treviglio, Bergamo, Italy) at 100 °C for 10 minutes to ensure microbial cell breakage. A subsequent centrifugation of the samples at 13,000 rpm for 5 minutes allowed to separate the aqueous phase from the biomass, leading to the formation of a precipitate at the bottom, and the supernatant containing the DNA, which was transferred into a new Eppendorf tube.

To evaluate the effective extraction of microbial DNA, a PCR with the universal prokaryotic primers 27f and 1492r was performed. Amplification via PCR was carried out using the following primers: 27f [5′-AGAGTTTGATCMTGGCTCAG] and 1492r [5′-GGTTACCTTGTTACGACTT3′-AAGTCGTAACAAGGTAACC]. The PCR was performed using a MyCycler Thermal Cycler (BioRad Laboratories) in a final volume of 25 µL for each reaction tube, according to the reaction conditions depicted in Figure 7.

The amplification was verified by electrophoresis by running the samples on a 1.5% (w/v) agarose gel in 0.5X Tris/Borate/EDTA (TBE) buffer containing 0.5 µg/mL GelRed® Nucleic Acid Gel Stain, 10,000X in water (Biotium, San Francisco, CA, USA). 5 µL of each amplicon were mixed with 2 µL of Loading dye and located into the dedicated wells of the electrophoresis gel. The electrophoretic run included the HyperLadder™ 1 kb (Meridian Bioscience, Cincinnati, Ohio, USA) as molecular weight standard and was carried out at 75 V for 3.5 h and visualized under UV light ($\lambda = 260$ nm).

The resulting images were visualized by a Canon Powershot G9 camera (Canon Italia S.p.A., Milano, Italy). Correct amplification was verified by comparison with the reference ladder. The amplicons were then shipped to Genewiz (Takaley, UK) for their purification and sequencing. Finally, a BLAST search was exploited to compare the obtained sequences (FASTA) with 16S rRNA sequences of type strains from GenBank database.
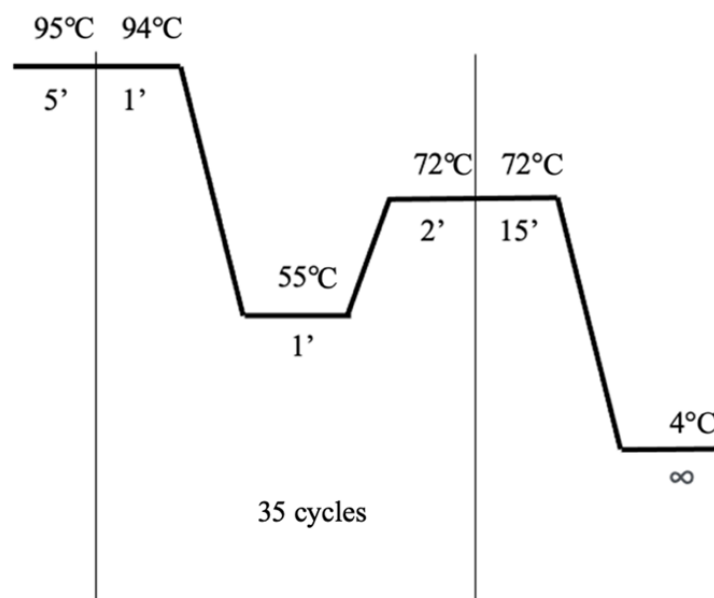
Figure 7: PCR amplification cycle.

2.5.2 Detection of hdcA gene of Gram-positive bacteria on microbial isolates

The reaction mix used in the PCR essay is shown in Table 3. The PCR reactions were carried out using the primer pair Hdc1 (50-TTGACCGTATCTCAGTGAGTCCAT-30) and Hdc2 (50- ACGGTCATACGAAACAATACCATC-30) designed by Fernandez et al. (2006) to amplify a fragment of 174 bp of the *hdcA* gene.

The PCR was conducted through a MyCycler Thermal Cycler (BioRad Laboratories) in a final volume of 25 μL for each reaction tube, according to the reaction conditions reported in Figure 8. The PCR amplification was verified by electrophoresis as previously described in paragraph 2.4.2. The electrophoretic run included the HyperLadder™ 100 pb (Meridian Bioscience, Cincinnati, Ohio, USA) as molecular weight standard. The analysis was carried out together with a blank, and the positive strain *Lactobacillus parabuchneri* DSM 5987.

Table 3. Reaction mix for hdcA gene detection.

Component	Initial concentration	Final concentration	Volume for each reaction tube
MyFi mix	1X	1X	12.5 μL
Hdc1	30 μM	900 nM	1 μL
Hdc2	30 μM	900 nM	1 μL

Water	Up to final volume (25 μL)
DNA	2 μL

95 °C 3'   95 °C 20''

72 °C 20''   72 °C 10'

58 °C 30''

15 °C ∞

40 cycles
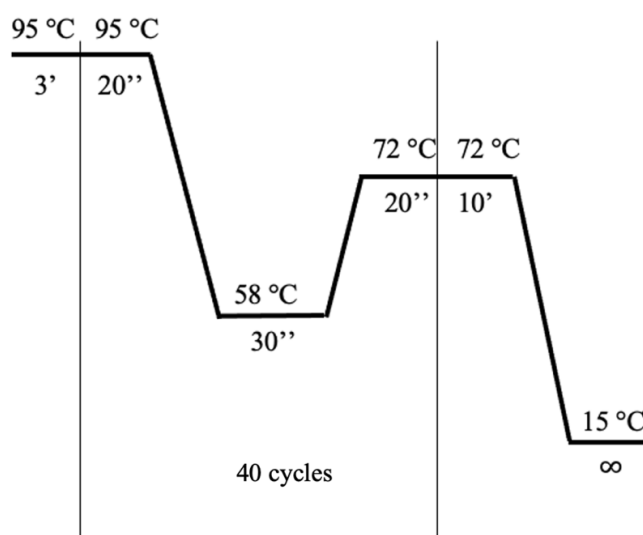
Figure 8. PCR amplification cycle.

2.5.2   *Assessment of antimicrobial activity*

The antimicrobial activity of the selected isolates was assessed through the agar well diffusion assay as previously described by Cardinali et al (2024). Prior to the test, the isolates were retrieved from frozen-stored suspensions and sub-cultured twice at 30 °C for 48 h; a third sub-culturing step was performed for 24 h. After the collection of an aliquot (500 μL) of the bacterial culture, the remaining broth cultures were centrifuged at 1610× g for 10 min. Then, the supernatant was neutralized (pH 7) with 0.1 N NaOH (AppliChem, Darmstadt, Germany). A filtration step on a sterile PES membrane filter of 0.22 μm pore size (Laboindustria S.p.A., Padova, Italy) was also performed. The Brain Heart Infusion (BHI) (VWR) soft agar (0.75%) growth medium was inoculated at a concentration of 2% (v v^{-1}) with the target microorganism *Listeria innocua* and subsequently poured into 90 mm diameter Petri dishes. After solidification, 200 μL sterile cone tips were used to create wells of ~50 μL. For each isolate, 3 wells were formed containing the following: (i) 50 μL of the sub-cultured suspension; (ii) 50 μL of the neutralized suspension; (iii) 50 μL of the filtered neutralized suspension. Finally, the Petri dishes were incubated at 37 °C for 24 h and checked for the presence of inhibition halos.

2.5.3   In-vitro EPS production

The selected isolates were tested for the production of EPS following a method previously described by Rampanti et al. (2024) with few modifications. First, the isolates were retrieved from cryo-protective suspensions and sub-cultured twice at 30 °C for 48 hours. Then, 5 µL-aliquots of each bacterial culture were added to the following solid media:

- MRS agar added with sucrose at a concentration of 80 g L^{-1} to promote the synthesis of homopolysaccharides (HoPS);
- MRS agar added with yeast extract (VWR Chemicals) (10 g L^{-1}), meat extract (VWR Chemicals) (10 g L^{-1}), lactose (Carlo Erba Reagents, Cornaredo, Italy) (20 g L^{-1}), and galactose (VWR Chemicals) (20 g L^{-1}) to promote the synthesis of heteropolysaccharides (HePS).

After an incubation of 48 hours at 30°C, colonies were classified as positive if they showed a ropy consistency (able to generate observable filaments with a sterile toothpick) or a mucoid aspect (visible shiny and slimy appearance). The analyses were performed in duplicate for each isolate.

2.5.4   Acidification Performance

To evaluate the kinetics of acidification by lactic acid bacteria in vitro, the procedure described by Rathore et al. (2012) was followed with minor adjustments. First, a sterile buckwheat bread extract (SBBE) liquid broth was prepared. In detail, 800 g of buckwheat flour were properly mixed with 1 L of water, 10 g of salt, and 25 g of fresh yeast. The dough was left to ferment at 30 °C for 2 hours, and subsequently baked at 220 °C for 30 minutes. After cooling, 400 g of chopped bread were suspended in 3.6 L of water (1:10 w v^{-1}) and sterilized by autoclaving at 121 °C for 20 min. The resulting mash was then centrifuged at 4000 rpm for 5 minutes. Finally, 20 ml-aliquots of supernatant were transferred into 50 ml Falcon tubes and sterilized by autoclaving at 121 °C for 20 min again to obtain a sterile liquid broth used in the following experiments.

The acidification performance of the isolates was assessed as previously detailed by Rampanti et al. (2024) with few modifications. First, the isolates were retrieved from the frozen stored suspensions and sub-cultured twice on the same medium used for the isolation. After growth, bacterial suspensions were centrifuged (4000 g for 5 min), and the cell pellets were resuspended in physiological solution (NaCl 0.9% w v^{-1}) in order reach a cell density of approximately 10^6 cells/ml. The acidification capacity of the lactic acid bacteria was then

tested by inoculating 20 ml of SBBE with 1% (v v^{-1}) of the of the cell suspension and by measuring pH after 4, 8, and 24 hours after inoculation.

2.5.5 Enzymatic Activities

Isolates with the best acidification performance were tested for key enzymatic activities, using the semi-quantitative, color-based micromethod API® ZYM (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Each API® ZYM (bioMérieux) strip is composed of 20 cupules containing synthetic substrates that are inoculated with the microorganism to be tested in a water suspension, this latter used to rehydrate the enzymatic substrates. The 20 cupules are designed to evaluate the activity of the following enzymes: 1 – control; 2 - alkaline phosphatase; 3 - esterase (C 4); 4 - esterase lipase (C 8); 5 - lipase (C 14); 6 - leucine arylamidase; 7 - valine arylamidase; 8 - cystine arylamidase; 9 - trypsin; 10 - alpha-chymotrypsin; 11 - acid phosphatase; 12 - naphthol-AS-BI-phosphohydrolase; 13 - alpha-galactosidase; 14 - beta-galactosidase; 15 - beta-glucuronidase; 16 - alpha-glucosidase; 17 - beta-glucosidase; 18 - N-acetyl-ß-glucosaminidase; 19 - alpha-mannosidase; 20 - alpha-fucosidase. The metabolic end-products produced during the incubation period were detected through coloured reactions revealed by the addition of reagents. Briefly, a suspension in API Suspension Medium (bioMérieux) (2 mL) of each isolate grown on MRS agar (Merck) was prepared according to a turbidity of 5–6 McFarland (bioMérieux). Sixty-five µL of the obtained suspension were used for the inoculation of each cupule of the API® ZYM (bioMérieux) strips with incubation for 4 h at 37°C. After incubation, 1 drop of ZYM A reagent (bioMérieux) and 1 drop of ZYM B reagent (bioMérieux) were added to each cupule until colour development (at least 5 min). For each cupule, a value ranging from 0 to 5 was assigned, corresponding to the colour developed: 0 corresponding to a negative reaction, 5 to a reaction of maximum intensity and 1, 2, 3, or 4 were intermediate reactions depending on the level of intensity (3, 4, or 5 being considered as positive reactions).

2.6 Statistical analysis

Significant differences in physico-chemical parameters and viable counts of Polish kvass-like beverage and Italian kvass-like beverage at different sampling times were determined by one-way analysis of variance (ANOVA) using the software JMP® Version 11.0.0 (SAS Institute Inc., Cary, NC, USA) and the Tukey–Kramer Honest Significant Difference (HSD) test (α = 0.05).

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Physico-chemical parameters

The results of pH and moisture content measurements of both Polish buckwheat kvass-like beverage (PK) and Italian buckwheat kvass-like beverage (IK) are reported in Table 4.

Table 4: pH and moisture content values of Polish and Italian samples.

	PK		IK	
	pH	Moisture content (%)	pH	Moisture content (%)
t_0	5.49 ± 0.01^a	96.94 ± 0.95^a	5.47 ± 0.01^a	96.63 ± 0.37^a
t_1	3.88 ± 0.03^b	96.34 ± 0.86^a	3.89 ± 0.01^b	97.36 ± 0.53^a
t_2	3.61 ± 0.01^c	96.11 ± 0.23^a	3.62 ± 0.01^c	96.11 ± 0.15^a
t_3	$3.56 \pm 0.01^{c,\ d}$	94.60 ± 0.42^a	$3.60 \pm 0.01^{c,\ d}$	95.55 ± 0.28^a
t_4	$3.55 \pm 0.04^{c,\ d}$	95.81 ± 0.46^a	$3.56 \pm 0.02^{d,\ e}$	97.51 ± 0.12^a
t_5	3.47 ± 0.01^d	97.21 ± 0.01^a	3.55 ± 0.00^e	97.21 ± 0.01^a

t_0 states for kvass immediately after inoculation of 24h-dough; t_1, t_2, t_3, t_4, t_5: kvass after one, two, three, four, and five days of fermentation in flasks. The results are expressed as mean ± standard deviation of two independent measurements. Within each column, mean values with different superscript letters are significantly different (P<0,05).

Concerning the Polish buckwheat kvass-like beverage (PK), the pH after inoculation (t_0) was 5.49 ± 0.01, whereas the moisture content at the same sampling time was 96.94 ± 0.95 %. Following 24 hours of incubation (t_1), the pH decreased to 3.88 ± 0.03, indicating the typical acidification process resulting from fermentation. These findings are consistent with those reported by Dlusskaya et al. (2008), where the pH of kvass produced from sourdough rye bread reached 3.4 after 24 hours. Statistically significant differences also emerged between the pH

values at t_1 and those at t_2, indicating a continued slight decrease after 48 hours. Similar pH values were observed at both t_3 and t_4 (approximately 3.55), indicative of a relative stability over time. The final pH (t_5) of PK beverage was 3.47 ± 0.01.

As for moisture content, no statistically significant differences were observed over time, with values ranging from 94.60 ± 0.42 to 97.21 ± 0.01.

A similar trend was observed for the Italian buckwheat kvass-like beverage (IK). In detail, the initial pH was 5.47 ± 0.01 and highly decreased after 24 hours of incubation, reaching 3.89 ± 0.01. After this initial rapid drop in pH, a slow and continuous decrease was observed from t_3 to t_5, reaching a final value of 3.55.

Also in this case, no statistically significant differences were observed for moisture content over time, with values ranging from 95.55 ± 0.28 to 97.51 ± 0.12.

The results of color parameters of kvass-like beverages over time are reported in Table 5.

Table 5: Color measurements of Polish and Italian samples.

	PK			IK		
	L*	a*	b*	L*	a*	b*
t_1	56.90 ± 0.66^a	0.13 ± 0.15^c	$7.63 \pm 0.51^{b,\,c}$	63.27 ± 3.04^b	-0.53 ± 0.15^c	5.23 ± 0.86^c
t_2	58.53 ± 0.81^a	2.40 ± 0.30^b	$9.07 \pm 0.47^{a,\,b}$	$66.60 \pm 0.70^{a,\,b}$	0.03 ± 0.15^b	$7.03 \pm 0.40^{a,\,b}$
t_3	57.67 ± 0.60^a	3.60 ± 0.36^a	10.50 ± 0.62^a	69.03 ± 1.36^a	1.33 ± 0.29^a	8.37 ± 0.23^a
t_4	56.85 ± 1.48^a	2.10 ± 0.30^b	8.34 ± 0.32^b	64.00 ± 0.60^b	$-0.10 \pm 0.10^{b,\,c}$	$5.63 \pm 0.31^{b,\,c}$
t_5	52.20 ± 1.01^b	0.73 ± 0.15^c	6.83 ± 0.70^c	63.27 ± 1.72^b	-0.60 ± 0.20^c	$2.07 \pm 0.90^{\,d}$

t_1: kvass after 1 day of fermentation; t_2, t_3, t_4, t_5: kvass after 2, 3, 4, and 5 days of fermentation in flasks. The results are expressed as mean ± standard deviation of three independent measurements. Within each column, mean values with different superscript letters are significantly different (P<0,05).

The Polish buckwheat kvass-like beverage (PK) showed lightness values ranging between 52.20 ± 1.01 and 58.53 ± 0.81. As for a* coordinate, the values ranged from 0.13 ± 0.15 to 3.60 ± 0.36. Wide variability was observed for both a* and b* parameters. Although statistically significant differences were observed among different times, the measured values indicate very low values tending toward redness. As for b* parameter, the values ranged between 6.83 ± 0.70 and 10.50 ± 0.62. Similarly to a*, a wide statistical variability was observed over time; however, all measured values indicate very low yellowness.

Slightly higher L* values were observed in the Italian buckwheat kvass-like beverage (IK) compared PK. For This sample, L* values ranged from 63.27 ± 1.72 to 69.03 ± 1.36. As for a* parameter, the values were in the range of -0.53 ± 0.15 and 1.33 ± 0.29, indicating neutral values in the red/green axis. As for b* parameter, the values ranged between 2.07 ± 0.90 and 8.37 ± 0.23. Similarly to a*, a wide statistical variability was observed over time; however, all measured values indicate very low yellowness.

3.2 Viable counts

The results of viable counting of kvass-like beverages are reported in Table 6. Overall, high loads of all microbial groups under investigation were observed in both Polish and Italian buckwheat kvass-like beverages.

Concerning PK sample, the loads of presumptive mesophilic lactococci ranged from 8.09 ± 0.11 to 8.91 ± 0.03 log cfu mL^{-1} over time. A gradual increase was observed until t_3, followed by a slight decrease until t_5. A similar trend was found for presumptive lactobacilli, with initial load of 8.20 ± 0.07 log cfu mL^{-1}, maximum load at t_3, attesting at 9.10 ± 0.12 log cfu mL^{-1}, and final load of 8.25 ± 0.01 log cfu mL^{-1}. Conversely, initial yeasts counts attested at 9.23 ± 0.09 log cfu mL^{-1}, and decreased gradually until the last sampling time, with statistically significant reduction to 7.79 ± 0.06 log cfu mL^{-1}.

As for the Italian buckwheat kvass-like beverage (IK), the initial load of presumptive lactococci was 7.62 ± 0.11 log cfu mL^{-1}; higher values were found starting from t_1 until t_5, ranging from 8.32 ± 0.07 and 8.66 ± 0.44 log cfu mL^{-1}. A similar trend was observed for presumptive lactobacilli; in detail, the initial load of this microbial group attested at 7.88 ± 0.01 log cfu mL^{-1} and increased starting from t_1 reaching the maximum load at t_3 (8.94 ± 0.09 log cfu mL^{-1}). The final count attested at 8.78 ± 0.08 log cfu mL^{-1}. Concerning yeasts, the results were similar to those found for PK sample. In this case, the maximum load was observed at t_1 (9.75 ± 0.20 log cfu mL^{-1}), gradually decreasing until t_5 reaching a final value of 8.46 ± 0.11 log cfu mL^{-1}.

Traditional kvass beverages are not subjected to heat treatment after fermentation, and thus contain high cell counts of viable yeasts and lactic acid bacteria (Osimani et al., 2014).

The results of this study are in accordance with those reported by Cardinali et al. (2021) for a buckwheat started beverage; similar loads of lactobacilli were found after 24h and 48h of fermentation. Kvass is similar to boza, a Turkish non-alcoholic cereal-based fermented beverage, with respect to the composition of the final product and the microorganisms involved in fermentation (Kabak et al., 2011). In the study conducted on unpasteurized commercial boza by Osimani et al. (2015), the loads of mesophilic lactococci and lactobacilli were comprised between 5.8 ± 0.01 and 6.8 ± 0.02 log cfu mL^{-1} and between 5.9 ± 0.02 and 6.5 ± 0.01 log cfu mL^{-1}, respectively. In the same study, the authors reported yeasts counts in the range of 4.9 ± 0.01 and 6.0 ± 0.01 log cfu mL^{-1}.

Table 6: Viable counts of Polish (PK) and Italian (IK)buckwheat kvass-like beverages.

	PK			IK		
	Presumptive lactococci	Presumptive lactobacilli	Yeasts	Presumptive lactococci	Presumptive lactobacilli	Yeasts
t_0	8.09 ± 0.11^b	8.20 ± 0.07^c	9.23 ± 0.09^a	7.62 ± 0.11^b	7.88 ± 0.01^b	9.08 ± 0.06^b
t_1	8.25 ± 0.02^b	8.31 ± 0.00^c	9.00 ± 0.18^a	8.66 ± 0.44^a	8.81 ± 0.03^a	9.75 ± 0.20^a
t_2	8.30 ± 0.06^b	8.82 ± 0.01^b	8.84 ± 0.17^a	$8.32 \pm 0.07^{a,b}$	8.90 ± 0.07^a	9.10 ± 0.02^b
t_3	8.91 ± 0.03^a	9.10 ± 0.12^a	8.96 ± 0.01^a	8.52 ± 0.23^a	8.94 ± 0.09^a	9.04 ± 0.08^b
t_4	8.74 ± 0.04^a	8.85 ± 0.03^b	8.76 ± 0.12^a	8.64 ± 0.06^a	8.83 ± 0.04^a	$8.81 \pm 0.09^{b,c}$
t_5	8.25 ± 0.03^b	8.25 ± 0.01^c	7.79 ± 0.06^b	8.46 ± 0.01^a	8.78 ± 0.08^a	8.46 ± 0.11^c

t_0 states for kvass immediately after inoculation of the 24h-dough; t_1, t_2, t_3, t_4, t_5: fermented kvass after the 1, 2, 3, 4, and 5 days of fermentation in flasks. Values are expressed as mean ± standard deviation of duplicate independent measurements. Within each column, means with different superscript letters are significantly different ($p < 0.05$)

3.3 Identification and characterization of lactic acid bacteria isolates

A total of 36 lactic acid bacteria (18 for each kvass-like beverage) were isolated, molecular identified, and characterized for safety and pro-technological traits.

The list of lactic acid bacteria isolated from PK and IK samples is reported in Table 7. In the same table, the results of the detection of the *hdcA* gene, assessment of antimicrobial activity against *L. innocua*, and production of EPS in-vitro are also reported.

In both PK and IK beverages, most of the isolates were identified as *Pediococcus pentosaceus*. In more detail, eleven *P. pentosaceus* were isolated from PK, twelve from IK.

In PK, *Leuconostoc mesenteroides* (4), *Pediococcus acidilactici* (2), and *Enterococcus durans* (1) were also identified. In IK, *Enterococcus durans* (3), *Leuconostoc mesenteroides* (2), and *Pediococcus stilesii* (1) were also found.

The knowledge of the microorganisms involved in the production of kvass and other cereal-based fermented beverages is important to better harness desirable traits of these non-alcoholic products. Research by Dlusskaya (2008) on commercial kvass demonstrated the presence of *Leuconostoc mesenteroides* and *Lactobacillus casei*. Wang et al. (2022) identified *Lacticaseibacillus casei* and *Lacticaseibacillus paracasei* among the predominant microflora in traditional barley malt kvass.

Pediococcus pentosaceus, a potential probiotic strain, plays an important role in the food industry and is often used as an additive to improve food taste and nutrition (Jiang et al, 2021). Previous studies confirmed that *P. pentosaceus* is commonly present in fermented products and could have active functions in product quality, food safety, and production efficiency by when prepared in a mixed fermenter with other bacteria (Jang e tal., 2015). *P. pentosaceus* in common in fermented foods such as pickles, dairy products, and sausages (Yining et al., 2021). The study conducted by Hu et al. (2021) demonstrated that *P. pentosaceus* associated with soybean milk can be a suitable strain for the development of functional plant-based beverages. *P.pentosaceus*, along with *L. mesenteroides*, predominates in the natural fermentation of non-alcoholic pearl millet beverages. Additionally, many studies have reported that *P. pentosaceus* has probiotic functions, including anti-inflammation, anti-cancer, antioxidant, detoxification, and cholesterol-lowering effects (Yining et al., 2021). As reported by Jideani et al. (2021), these identified LAB strains could potentially be developed as starter cultures for industrial production. Conversely, *Enterococcus durans* appears to have been detected for the first time in this type of product.

Table 7: Identification and characterization of lactic acid bacteria isolated from Polish and Italian buckwheat kvass-like beverages.

Isolation source	Isolate Code	Closest relative	*hdcA* gene	Antimicrobial activity	EPS	
					Sucrose-depentent	Sucrose-independent
PK	P3	*Pediococcus pentosaceus*	+	-	-	-
	P4	*Pediococcus acidilactici*	-	-	-	+
	P5	*Pediococcus pentosaceus*	+	-	+	-
	P6	*Pediococcus pentosaceus*	-	-	+	+
	P7	*Pediococcus pentosaceus*	+	-	-	-
	P8	*Pediococcus pentosaceus*	+	-	-	-
	P9	*Pediococcus acidilactici*	-	-	-	-
	P10	*Pediococcus pentosaceus*	+	-	+	+
	P11	*Leuconostoc mesenteroides*	-	-	+	+
	P12	*Pediococcus pentosaceus*	+	-	-	-
	P13	*Pediococcus pentosaceus*	+	-	-	+
	P14	*Pediococcus pentosaceus*	+	-	+	-
	P15	*Enterococcus durans*	-	-	-	-
	P16	*Pediococcus pentosaceus*	-	-	-	-
	P17	*Pediococcus pentosaceus*	+	-	-	-
	P18	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	-	-	-	-
	P19	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	+	-	+	-
	P20	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	-	-	+	-
IK	I1	*Pediococcus pentosaceus*	-	-	-	-
	I2	*Pediococcus pentosaceus*	+	-	+	-
	I3	*Pediococcus pentosaceus*	+	-	+	-

I4	*Pediococcus pentosaceus*	-	-	+	-
I5	*Pediococcus pentosaceus*	-	-	+	-
I6	*Pediococcus pentosaceus*	+	-	+	-
I7	*Pediococcus pentosaceus*	+	-	-	-
I8	*Pediococcus pentosaceus*	+	-	-	-
I9	*Pediococcus pentosaceus*	+	-	-	-
I10	*Pediococcus pentosaceus*	-	-	+	-
I11	*Pediococcus pentosaceus*	-	-	+	-
I12	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	-	-	+	-
I13	*Leuconostoc mesenteroides subsp. jonggajibkimchii*	-	-	-	-
I14	*Pediococcus pentosaceus*	-	-	-	-
I15	*Pediococcus stilesii*	+	-	-	-
I16	*Enterococcus durans*	+	-	+	-
I18	*Enterococcus durans*	+	-	-	-
I19	*Enterococcus durans*	-	-	-	-

3.3.1 Detection of hdcA gene

The presence of the *hdcA* gene was revealed in ten lactic acid bacteria isolated from PK, specifically P3, P5, P7, P8, P10, P12, P13, P14, P17, P19, and nine isolates from IK, specifically I2, I3, I6, I7, I8, I9, I15, I16, I18. Most of the positive isolates were identified as *P. pentosaceus*. One *L. mesenteroides* subsp. *jonggajibkimchii*, one *E. durans*, and one *P. stilesii* were also positive.

The *hdcA* gene in Gram-positive bacteria encodes a pyruvoyl-dependent decarboxylase responsible for converting the amino acid histidine into histamine (Hilbig et al., 2019). Histamine poses a significant food safety risk, as high levels can lead to health issues such as hypotension or hypertension, nausea, headache, rash, dizziness, cardiac palpitation, and emesis (Cosansu, 2009). The detection of histamine-producing lactic acid bacteria represents a preventive strategy to avoid their use as starter or adjunct cultures, thus protecting consumer health (Di Renzo et al., 2023). Fermented products typically contain the highest concentrations of histamine, significantly more than non-fermented foods.

The absence of the *hdcA* gene in the other analyzed isolates is a favorable trait, making them suitable candidates for potential starter cultures in the production of kvass and other cereal-based fermented beverages.

3.3.2 Antimicrobial activity

Another important safety consideration when selecting lactic acid bacteria for use as starter or adjunct cultures is their antimicrobial activity against spoilage or pathogenic bacteria (Di Renzo et al., 2013).

None of the tested cultures showed antimicrobial activity against *Listeria innocua* (Table 7). The production of antimicrobial compounds by lactic acid bacteria is well-documented in scientific literature (Perez et al., 2022). LAB exert a preservative effect by producing various antimicrobial metabolites, including bacteriocins, which are among the most potent bacterial inhibitors. Since most bacteriocin-producing LAB are natural food isolates, they are ideally suited for food applications (Deegan et al., 2006). Fugaban et al. (2022) demonstrated that *Pediococcus acidilactici* and *P. pentosaceus* produce bacteriocins with high specificity against *Listeria.* However, the ability of lactic acid bacteria to produce such molecules is highly strictly strain-dependent and involves the expression of numerous genes related to the processing of precursor peptides, secretion, autoimmunity, and regulation of their production (Perez et al., 2022).

3.3.3 In-vitro EPS production

The in vitro assay used in this study provides a rapid and effective method for the preliminary selection of EPS-producing lactic acid bacteria as potential adjunct cultures. The results are shown in Table 7. Eleven *P. pentosaceus* isolates, four from PK (P5, P6, P10, P14), and seven from IK (I2, I3, I4, I5, I6, I10, I11), respectively, were able to produce EPS on MRS agar added with sucrose (HoPS). Four *L. mesenteroides* isolates (P11, P19, P20 from PK and I12 from IK) showed their ability to produce HoPS. Moreover, three *P. pentosaceus* isolates (P6, P10, P13), one *P. acidilactici* (P4), and one *L. mesenteroides* (P11) produced HePS None of the lactic acid bacteria isolated from IK exhibited in-vitro HePS production. This findings are consistent with the study conducted by Smitinont et al. (1999), where LAB isolated from various fermented foods were screened for their EPS production, with *P. pentosaceus* strains showing particularly high EPS yields. Abedfar et al. (2018) further investigated the role of *P. pentosaceus* in EPS synthesis, finding that most LAB isolates from wheat bran sourdough attributed to *P. pentosaceus* were EPS producers.

Exopolysaccharides (EPSs) are high molecular weight polymers biosynthesized by a wide range of bacteria, including lactic acid bacteria. EPSs can be classified into two groups: homopolysaccharides, which are composed of one type of monosaccharide, and heteropolysaccharides, which consist of repeating units of two or more types of monosaccharides. EPSs produced by LAB have recently garnered significant attention due to their health benefits, which include antitumor, anti-ulcer, antioxidant, cholesterol lowering, and immune-stimulating activities (Sanalibaba et al., 2016; Surayot et al., 2014). Additionally, some EPSs produced by LAB can function as viscosifiers, thickeners, emulsifiers, or stabilizers in the food industry (Bajpai et al., 2015). While some EPSs form biofilms that pose hygiene issues, other play a crucial role in improving the rheology, texture, mouthfeel of fermented foods (Sasidharan et al., 2015). The in-situ production of EPS in fermented plant-based beverages has been extensively studied for its potential to improve sensory properties (Jurášková et al., 2022). Moreover, polysaccharides have gained interest in the production of higher-quality gluten-free products (Krocz et al., 2021).

Further studies are needed in the field of cereal-based fermented beverages in order to gain a comprehensive understanding and to characterize potential starters and adjunct cultures.

3.3.3. Acidification performance

The initial pH of the sterile buckwheat bread extract (SBBE) before inoculation was 6,87. The results of acidification performance of lactic acid bacteria isolated from PK and IK are reported in Figure 9 (panel a and b, respectively).
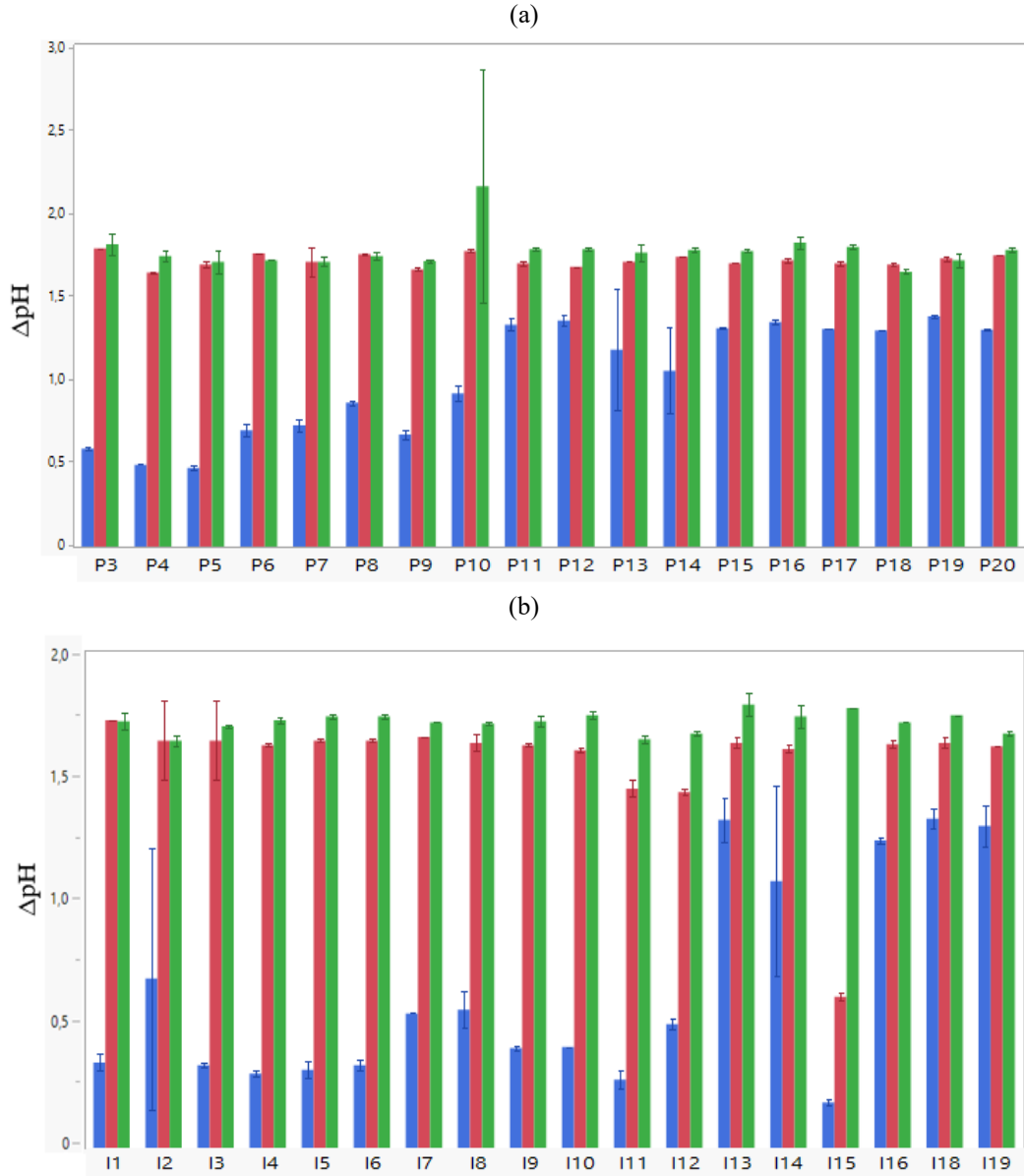
(a)

(b)

Figure 9: Acidification performance of lactic acid bacteria isolated from kvass-like samples after 4 h (blue bar), 8h (red bar), and 24 h (green bar) of fermentation. Panel a: isolates from PK; panel b: isolates from IK.

In more detail, regarding PK (panel a), isolates P11, P12, P16, and P19 showed the highest acidification performance after 4 hours, with ΔpH values close to 1.5. After 8 hours, the isolates maintained a similar trend, reaching very similar values. Isolate P10 (*P. pentosaceus*) exhibited the best acidification after 24 hours. Isolates P3, P11, P12, and P16 also showed good acidification activity over 24 hours.

Concerning IK, after 4 hours, there was a noticeable differentiation among the values, with isolates I13, I16, I18, and I120 exhibiting the highest acidification levels, with ΔpH values ranging between 1.28 and 1.34. Conversely, after 8 hours, the most performing isolates were I1, I2, I3, I5, and I6, while I15 showed the lowest value. Finally, after 24 hours, I13 emerged as the isolate with the best acidifying performance, notable alongside I5, I6, I10, I14, and I16. The acidification activity is crucial for evaluating lactic acid bacteria as potential starters for producing fermented foods. To determine if LAB are effective and can adequately acidify the substrate, it is necessary to conduct acidification performance tests. This is essential for ensuring product quality and meeting safety thresholds.

Cardinali et al. (2021) observed that strains with the best performance in terms of acidification rate (pH < 5.5 after 8h fermentation) and acidification extent (pH < 4.8 after 24h fermentation) could be selected for the formulation of starter cultures.

3.3.4. Enzymatic Activities

The results of the semi-quantitative assessment of enzymatic activities using the API® ZYM system are presented in Figure 10. This test was conducted on the isolates that showed the best acidification activity, including three isolates from IK (I1, I13, I14) and four from PK (P11, P16, P18, P20).

In detail, none of the tested isolates exhibited positive reactions for alkaline phosphatase, esterase (C4), esterase lipase (C14), lipase (C14), trypsin, alpha-chymotrypsin, alpha-galactosidase, beta-glucuronidase, N-acetyl-β-glucosaminidase, alpha-mannosidase, and alpha-fucosidase.

Notably, isolate I1 showed strong activity for leucine arylamidase and valine arylamidase, similarly, to isolates I14 and P16. For beta-glucosidase, isolates I13, P11, P18, and P20 exhibited the highest activity. All isolates showed weak activity for naphthol-AS-BI-phosphohydrolase.
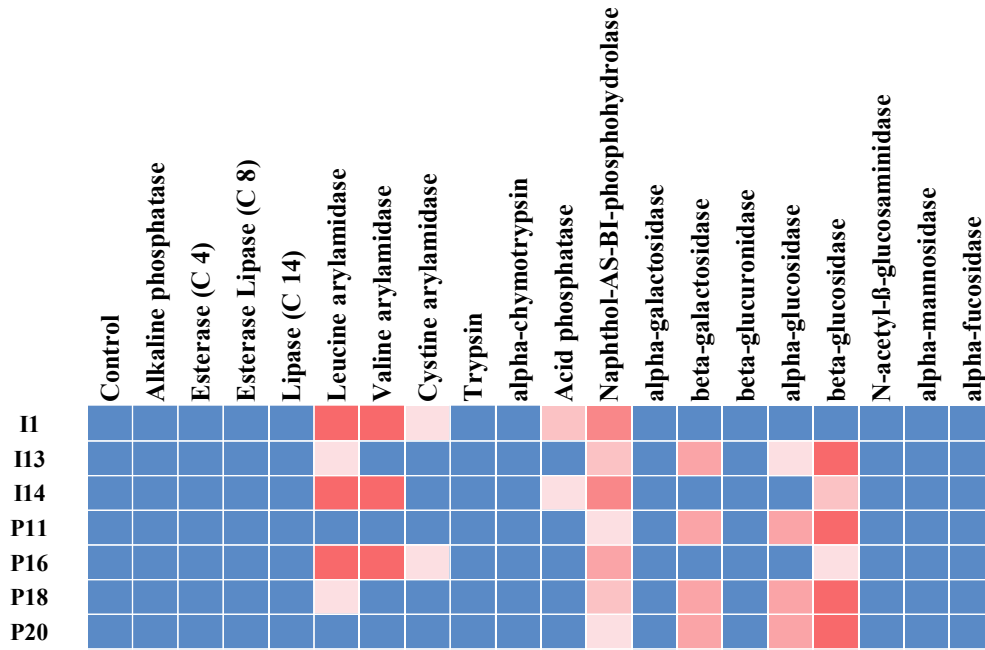
Figure 10: Semi-quantitative assessment of enzymatic activities of lactic acid bacteria isolated from Italian and Polish buckwheat kvass-like beverages.

CONCLUSIONS

The aim of this study was to evaluate a production process for a non-alcoholic fermented beverage known as kvass, using stale bread made with buckwheat *(Fagopyrum esculentum)*, and to investigate the microflora of the final product for the development of starter cultures.

Cereal-based fermented beverages are recognized for their numerous advantages, including beneficial effects on human health, gluten-free nature, and potential positive environmental impact through the reuse of stale bread, that would otherwise be considered food waste.

Pediococcus pentosaceus and *Leuconostoc mesenteroides*, the main lactic acid bacteria isolated in this study, have not yet been evaluated in the literature as starter cultures for kvass production. Their characterization allowed for the selection of isolates with the most promising traits. Only isolates I1, I13, I14, P11, P16, P18, and P20 exhibited promising pro-technological traits for use as starter cultures, primarily due to their acidification performance. Many isolates from both Polish and Italian buckwheat kvass-like beverages produced HoPS, including P5, P6, P10, P11, P14, P18, P20 and I2, I3, I4, I5, I6, I7, I10, I11, I12, I16. None of the tested isolates produced bacteriocins against *Listeria innocua*. Furthermore, many isolates showed the presence of the *hdcA* gene, highlighting their unsuitability for the production of fermented beverages.

This study marks an initial step towards transitioning from traditional home methods to industrial-scale beverage production through controlled fermentation process, improving the safety and quality of the final products.

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