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Ph.D. School in Agri-Food Sciences, Technologies and Biotechnologies

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XXXII Cycle

INNOVATIVE ACETIFICATION PROCESESS AND PRODUCTS DEVELOPED AT ACETAIA SAN GIACOMO COMPANY

Ph.D. Candidate: Andrea Bezzecchi

Tutor: Dr. Maria Gullo

Dean of Ph.D. School: Prof Alessandro Ulrici

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Abstract of the thesis

The Italian production of vinegars has recently been involved in a regulatory revision that has identified, alongside the industrial production, the so-called "static fermentation and long-maturation" vinegars, also known as "artisanal vinegars". The recognition of this latter category reflects the radical differences in which the production of vinegars is divided. On the one hand the industrial production, which interfaces mainly with production yield issues; on the other hand the artisanal production which, carried out in low-tech contexts, is characterized by longer time for obtaining products, respect to the industrial production. However, the production of artisanal vinegars, despite having strong production limits, shows wide margins of innovation and the market is becoming more and more multi-segmented and specialized where the consumer are constantly participating in the value chain becoming co-creator of value (service and products). In this industrial doctorate project, born from the needs of the San Giacomo Company, starting from an analysis of the products / processes consolidated by the Company, the following topics were developed:

- a kit and a working procedure for the production of vinegar by not specialized users. To obtain the kit, consisting of devices for the production, monitoring and starter cultures of acetic acid bacteria, experiments were carried out testing raw materials in different fermentation conditions. The results demonstrated the feasibility of processes starting from raw materials having a variable composition.
- Subsequently, cheese whey, the main waste of the dairy industry, was considered for the production of condiment / beverages with health and environmental sustainability attributes. Cheese whey was tested in combination with kombucha tea and kefir to obtain non-alcoholic sour healthy drinks or low acid condiment for food preparation. The approach used in this study represents a highly promising strategy for obtaining condiment or non-alcoholic fermented beverages.

Starting from the consideration that vinegar and rice beverages are widely consumed in Asian countries, thanks to their healthy connotations, in this work rice syrup was identified as fermentable raw material for the production of new vinegars / condiments. From starter cultures developed at laboratory scale from selection of company's mixed cultures and tested at a prototype scale, vinegars of different composition were obtained, which can be optimized as finished products or ingredients for the production of condiments.
- During a prior overview about Acetic Acid Bacteria and their heterogeneous application, a study

was carried out on bacterial cellulose due to its contradictory role in vinegar production. It represents an undesired product of acetic acid fermentation conducted with mixed cultures, since it leads a decrease in acetic acid and not appreciated sensory characteristics of vinegar. However, in some emerging products, the presence of bacterial cellulose is considered a positive attribute. Furthermore, the bacterial cellulose is considered an interesting biopolymer by the biotechnology industry due to the wide application possibilities in the food and medical fields. On the bases of these observation, the ability to produce cellulose by an acetic acid bacterium strain was tested, buy cultivation in the presence of different carbon sources and the genome sequencing was conducted. The results showed that the strain is highly versatile in the production of cellulose and is a good candidate for industrial uses. The results presented in this thesis work prove that the artisanal production of vinegar has high margins of innovation. These innovations are all based on application of the static superficial fermentation process which, using mixed or selected bacterial cultures, allow to diversify the products, enlarging the market offer and reach an higher positioning.

RIASSUNTO

Il settore merceologico italiano degli aceti è stato recentemente interessato da una revisione normativa che ha identificato, a fianco della produzione industriale, i cosiddetti aceti "da fermentazione statica e lunga maturazione", che possono legittimamente definirsi "artigianali".
Il riconoscimento di questa categoria rispecchia le radicali differenze e i due segmenti ben distinti in cui la produzione di aceti si divide. Inoltre, riconosce di fatto, l'esigenza di una nicchia sia di mercato che produttiva.
Tali nicchie sono il fenomeno più consistente che sta investendo mercati maturi, dove segmentazione e specializzazione dell'offerta, attraverso la capacità di veicolare nuovi significati, sia funzionali che simbolici, sono le strategie fondamentali che le aziende devono essere in grado di implementare.
La produzione di aceto artigianale pur presentando forti limiti di produzione, mostra ampi margini di innovazione, soprattutto nell'ottica di una forte segmentazione di mercato che vede il consumatore finale entrare sempre di più nella co-creazione del valore potendo diventare esso stesso produttore se dotato delle procedure e dei mezzi idonei.
In questo progetto di dottorato industriale, nato dalle esigenze dell'Azienda San Giacomo, partendo da un'analisi dei prodotti/processi consolidati dall'Azienda, sono stati sviluppate le tematiche di seguito riportate:
- È stato sviluppato un kit ed una procedura di lavoro per la produzione di aceto da parte di utenti non specializzati. Per l'ottenimento del kit, costituito da dispositivi per la produzione, monitoraggio e colture starter di batteri acetici, caratterizzati dall'economicità e semplicità d'utilizzo, sono stati effettuati esperimenti per testare materie prime in differenti condizioni di fermentazione. I risultati hanno dimostrato la fattibilità di processi a partire da materie prime a composizione variabile.
- Successivamente è stato considerato il siero di latte, principale scarto dell'industria casearia, per la produzione di condimenti/bevande con carattere salutistico e di ecosostenibilità. Il siero di latte è stato testato in combinazione con kombucha tea e kefir per ottenere bevande non alcoliche e a bassa acidità. L'approccio impiegato rappresenta una strategia altamente promettente per l'ottenimento di bevande fermentate non alcoliche ma anche, per ottenere condimenti a bassa acidità per preparazioni alimentari.

Partendo dalla considerazione che l'aceto e le bevande di riso sono ampiamente consumati nei paesi asiatici grazie alla loro connotazione salutistica, e che il settore della ristorazione è particolarmente ricettivo rispetto ad aceti diversi da quelli ottenuti dal vino, con basso tenore di acidità, in questo lavoro si è individuato lo sciroppo di riso italiano come materia prima fermentescibile per la produzione di nuovi aceti/condimenti. Dalle colture starter, isolate in azienda, sviluppate su scala di laboratorio e testate in scala prototipale, sono stati ottenuti aceti a diversa composizione che possono essere ottimizzati come prodotti finiti o ingredienti per la produzione di condimenti.

- Durante il preventivo studio relativo ai Batteri Acetici e il loro variegato impiego in processi fermentativi, stato effettuato uno studio sulla cellulosa batterica per il suo ruolo contraddittorio nella produzione di aceto. Rappresenta un prodotto indesiderato della fermentazione acetica condotta con colture miste poiché comporta una diminuzione in acido acetico ed impartisce caratteristiche sensoriali non apprezzate all'aceto. Tuttavia in alcuni prodotti artigianali emergenti, la presenza di cellulosa batterica è considerata un attributo positivo. Inoltre la cellulosa batterica richiama un forte interesse da parte dell'industria biotecnologica per le ampie possibilità di applicazione in campo alimentare e medico. Sulla base di tali premesse, è stata valutata la capacità di produzione di cellulosa da parte di un batterio acetico, sottoposto a coltivazione in presenza di diverse fonti di carbonio ed è stato condotto il sequenziamento del genoma. I risultati hanno mostrato che il ceppo è altamente versatile nella produzione di cellulosa e risulta un buon candidato per usi industriali.

I risultati presentati in questo lavoro di tesi provano che la produzione artigianale di aceto "artigianale" presenta alti margini di innovazione. Tali innovazioni sono principalmente legate alle fasi di fermentazione che condotte con colture miste o colture selezionate permettono da un lato di adottare procedure differenti dall'altro di diversificare i prodotti.

AIMS, OBJECTIVES AND STRATEGIES

This PhD thesis is the result of a project born from the need of Acetaia San Giacomo to develop new processes and products in the light to increase the innovation capability of the company and differentiate the offer.
According to the guidelines of Industrial PhD programme of the University of Modena and Reggio Emilia, the project has been developed at Acetaia San Giacomo and at the Department of Life Sciences (Food Microbiology/UMCC laboratory) between the years 2017 and 2019.
The collaboration between Acetaia San Giacomo and the Department was first established in 2014 for developing the project "*Produzione di un sistema di acetificazione innovativo - Acquisizione di servizi di ricerca e sperimentazione*" (Contributo Regione Emilia Romagna. Decreto del Commissario n. 393 del 13/03/2014) of Acetaia San Giacomo Company, in collaboration with the Department of Life Sciences of the University of Modena and Reggio Emilia.
In this project main technological needs and perspectives for innovation have been highlighted; all raised up issues have been analyzed and constitute the frame of the Industrial PhD thesis.

The general aim of this thesis is to investigate if the application of the static acetic fermentation method could represent a possible competitive advantage for small companies in search of higher market position. The *ratio* of the investigation starts from an apparent counterintuitive point of view. Technologically speaking, efficiency (meant as time saved, increased yield, continuity, standardization) is the main driver. However, from a market perspective, starting from company's experience, we noticed how market segmentation face different needs of values, added to the product.
In western countries, where the wine sector has the capacity to intercept hedonic needs, becoming, in some cases, a status symbol, vinegar has been culturally limited to one of the defects or problems that can afflict wine. However, in other countries (mainly in Asia), vinegar has met excellent considerations for centuries due to the healthiness of the product. This vision is affecting the new generations and both the market and the perception of the product is changing.
In this study, we have tried to leverage a fermentation process which, if conducted in a structurally opposite way to the industrial one, is able to raise the perceived value. Regardless of the final result in terms of organoleptic (as they are highly subjective) and other features (quality certifications such as organic, PDO, health claim or others) that even industrial productions can boast. So, a rare case where a biochemical process can be a competitive advantage per se.

In **Chapter 1**, the conceptual framework starting from market and company analysis and regulatory context, was designed as premises of the entire research.

Through conceptual framework designing, which resulted in a matrix correlating market positioning/technology involved/added value, an empty space suitable of innovation, has been defined.

For the company's productive set up (entirely based on static superficial acetic fermentation and long maturation vinegars and condiments) that space could be reach with two (incremental) innovative drivers.

One drivers require an adapting and shifting of productive offer in order to match emergent needs. The features implemented on the innovative products or processes, should face the ability to respond to new consumer's desires. But a simple shifting and adapting of the offer not necessary give a competitive advantage. Therefore, that advantage can be given through a positioning asymmetry brought by a higher perceived value. We defined the static superficial acetic fermentation, the artisanal method, an implicit feature able to convey that symbolic value that also the industries are aiming to use in claiming their product.

In **Chapter 2**, an overview on bacterial cultures producing acetic acid and their use in fermentation processes, is provided.

General characteristics and traits of Acetic Acid Bacteria (AAB) were analyzed and a state of the art of the vinegar production techniques was done.

Starting from the attitude of certain strains of AAB in producing Bacterial cellulose, which is a controversial feature of their metabolic activity, a deeper analysis of an AAB strain was performed. The objective of this part of the study was to evaluate the ability of K1G4 strain in producing bacterial cellulose from different carbon sources and to assess the genome sequencing with special focus on bacterial cellulose related genes.

From this research, the manuscript entitled *Genome sequencing and phylogenetic analysis of K1G4: a new Komagataeibacter strain producing bacterial cellulose from different carbon sources"* was produced and submitted to the Journal "Biotechnological Letters" on October, 1, 2019, as documented by the submission letter attached to the Thesis.

Chapter 3, is focused on the test performed to design a user-friendly kit for domestic production of vinegar by not specialized users.

The incremental innovation is referred to the resizing and adapting the static acetic fermentation process to an unusual environment. Adapting of the process took as premises the targeted customer's needs. In particular, affordability, ease of use, basic knowledge gap, simple control system, were the main drivers of the research.

Tests on commercial wine (as typical fermentative substrate) with and without selected AAB cultures, were performed in order to understand which was the best start-up condition.

Use of selected cultures in start-up were identified as the most reliable condition. Therefore, tests on lyophilized strain of AAB were performed (see Appendix).

A commercial and affordable pHMeter was tested in order to understand which part of the process could be applied to. In this part of research tests on different small container (flask, beaker) with and without aerator, were performed to understand the viability of a small scale submerged fermentation process.

Finally, in this chapter we proved the feasibility to create a set of basic knowledge and affordable tools able to allow not professional users to produce vinegar in uncontrolled environment.

The process innovation related to the designing and adapting the kit for domestic vinegar production by not specialized users, has already provided interest from media, private individual and restaurateurs (see Appendix).

In **Chapter 4**, an unconventional raw material has been evaluated as possible fermentative media for production of innovative vinegar products.

The purpose of the present study arose from the need to valorize the cheese whey as a waste byproduct, together with the fulfilment of the current market requests concerning beverage with functional healthy benefits, or innovative condiments with unusual flavor profile.

Kombucha and kefir were adopted as bacterial cultures performing a series of scaling fermentation to reach the desired parameters.

The first feedback from company's customers, identify the best application of the innovative product, as seasoning for meat marinating (artificial tenderization made by acid solution).

In **Chapter 5,** stating the emerging needs for new flavors, even if related to different food cultures (mainly eastern), tests using Italian rice syrup were performed.

The already saccharified rice syrup is a staple raw material for industrial production of rice vinegar. The application of static method with selected AAB cultures, isolated from mixed culture in static fermentation from the company, is the innovative incremental activity performed.
Six biofilm from different raw materials media from barrels of 220 Lt capacity at the company, were collected.
In laboratory, the isolation and cultivation in fermented rice broth of the two more active strains were performed. A scaling up process in fermented rice broth (Ethanol 7.5% v/v) was performed subsequently at the company.
The final result of static acetic fermentation ends with a level of titratable acidity detected superior of 5% (w/v). The final parameters are coherent with the vinegar category requirement, and revealed the attitude of the selected raw material to be fermented with static superficial process.

Chapter 1

TO DEFINE THE FIELD OF APPLICATION OF THE RESEARCH

CHAPTER 1. TO DEFINE THE FIELD OF APPLICATION OF THE RESEARCH

1.1 Introduction

The aim of this chapter is to define the conceptual framework of this industrial PhD thesis (Figure 1), first defining the scope of investigation, then trying to assess a possible application of the results to a micro-small agrifood business.

The method followed a bottom-up approach applied to a "marketing-first" analysis:

- market assessment with focus on vinegars consumption within different channels, understanding the trends and new upcoming needs;
- investigate a normative and productive space that needs innovation;
- define "innovation" in the field previously pointed out;
- define the "added value" in general and those expected from the consumers, in response to the emerging needs;
- identify the field of application according to the Italian's manufacturing situation, the company productive set-up and its market positioning (competitive analysis).

The ultimate goal is to asses a first horizontally investigation about new products and processes that are able to fit the identified needs for a specific market segment.

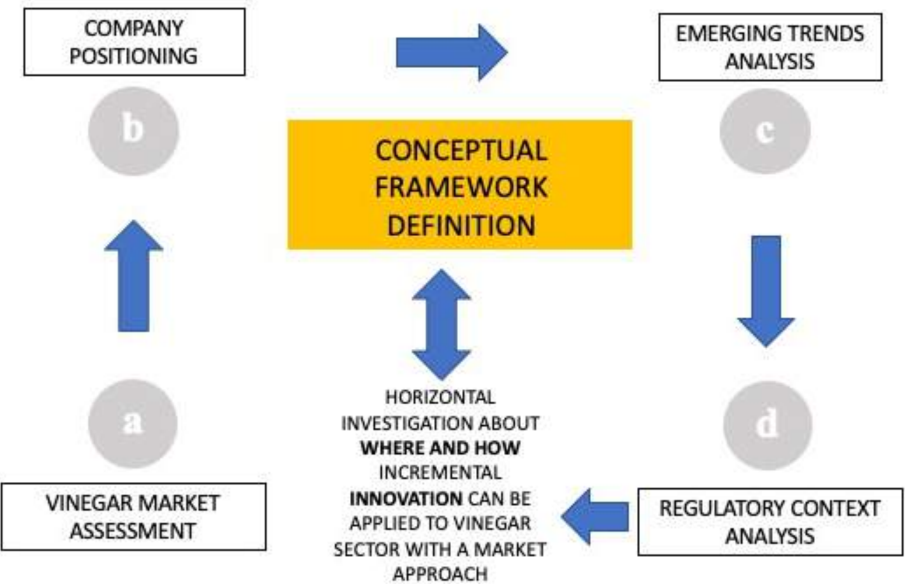


Fig. 1. Research framework definition.

1.2 Overview on vinegar market

The method used in this assessment, starts from the analysis of the vinegar consumption in different sectors. The vinegar business is not equally monitored in the different countries, so due to the lack of data regarding this specific branch of the market, an integrated approach with the market trends and actual market data from the company was necessary.

The global vinegar market has reached values worth around USD 1.26 billion in 2017 growing at a rate of 2.1 % during 2010-2017 (https://www.imarcgroup.com/vinegar-manufacturing-plant) and is further expected to reach a value of around USD 1.50 Billion by 2022.

Geographically, Europe represents the biggest market for vinegar (more than half of the total global market share) followed by North America and the Asia Pacific region (Morin & Lees, 2018). In 2016, balsamic vinegar exhibited a clear dominance with the majority of market share. The use of vinegar is increasing in different cuisines, which results in increasing demand. Growing populations, rising disposable incomes, increasing health consciousness among consumers and the food and beverage Vinegar industry are the main driving factors of the vinegar market. It is expected that the global vinegar market will witness growth both in terms of revenue and volume during the following years. Growth will come from changing consumer lifestyles and preferences. The interest in cooking gourmet and ethnic foods have increased among many consumers, thus prompting the sales of various dressings, most of which use vinegar as one of the key ingredients.

1.2.1 Different sectors of vinegar consumption

The food, and so the "vinegars", consumption is divided into "Food retail" and "Food Service" sectors, nowadays known as Out-of-Home (or Ho.Re.Ca. in broader way).

The prevalence so far is for the Food Retail (Food Market segmented in Iper, Super, Discount, Medium sized store from 100 to 400 square meters) for approximately 65% of the market share although, starting in 2013, the "Out of Home" is recording constant increases (source: Pwc). It seems that the tendency is to get closer to other countries like United States, where relations are reversed.

1.2.2 The "Food Retail" sector – the Italian market

Vinegars as ingredient can claim the highest penetration rate among Italian families.

The Italian vinegar sector in fact continues to grow, according to latest researches in Food Retail market (Table 1). If the core of the business is what is considered the "classic" product (wine vinegars without specification in grape's varietals and their origin), triggering the entire sector by volume and value, the exploit of apple vinegar stands out, from which very promising sub-segments are born, such as that offered as drinks, following the success of healthy and natural trends.

Balsamic vinegar, like other "Traditional" vinegars, on the other hand, marks the pace, confirming an ongoing trend which is not from today, and is consoled with the successes that it continues to reap on international markets, to which more than 90% of production is destined (source: ISMEA Qualitavita report 2018).

Special "vinegars" are considering those made with particular wines (DOC and DOCG) or specific grapes varietals. They have a downward trend as well.

Segments	Sales (value in €)	% var.	Sales (quantity in lt.)	% var.	Average price (€/lt)	% var.
Classic Vin.	59.737.300	+4,5	68.305.225	+4.1%	0.87	+0.4
Balsamic PGI	44.463.924	-1,6	6.803.511	-3.1	6.54	+1.6
Apple Vin.	23.525.737	+9,7	7.792.397	+6.9	3.02	+2.6
Bals. Cream	13.563.336	-1,5	1.452.120	-1.7	9.34	+0.1
Special Vin.	5.661.133	-4,8	2.279.297	-6.0	2.48	+1.2
Total vinegar	146.951.431	+2,4	86.632.549	+3.4	1.70	-0.9

Italy total – source: Nielsen

Table 1. Segments size and trends – Italian Food Retail sector from 25.02.2018 to 24.02.2019.

The research doesn't monitor the "normal trade" (independent store below 100 square meters). The latter channel trades more premium priced products in comparison with the monitored channel where the average price for the considered category is 1,70 Euro/l.
The analysis revels that there is a decrease of the "Traditional" product (perceived as "Traditional" despite the production technique) like PGI Balsamic Vinegar of Modena, Balsamic Cream and the "Special Vinegars".
In the "condiments category", apple vinegar is undoubtedly one of the sub-category that has registered the best sales performance in recent years; third segment by value in terms of value in the Italian vinegar market, with + 11.5% in 2017 and 9.7% in 2018 as shown in table 1, marked the greatest growth trend in value in the retail channel.
The biggest PGI Balsamic Vinegar factories are remodeling their productive asset in shifting toward a new production based on apple as main ingredient, able to match upcoming needs from the customers.
Usually the sales tendency in the large-scale Food Retail, are the late reflection of a trend already underway, brought by a generational change that is modifying the customer expectations and habits.
Traditional products, especially those with long aging like Traditional Balsamic DOP are keeping they market share but new and innovative products can be used to approach new market, whose are always looking to differentiate their offer. With regards to the Food Retail, it seems that the "novelty", the "new", can be factors of interest for an increasingly attentive public, which is also open to trends that go beyond the traditional/typical products that for years have been driving the entire sector.

1.2.3 The "Food Service" sector

In 2016 over 329,000 food service companies were active in Italy. It is a highly-fragmented market with a greater quota owned by micro and small business (according to the EU definition).
Due to lack of precise information and numbers about vinegars consumption in Food Service sector, the research take action from an analysis of the market progress, assuming the vinegars as basic ingredient, so strictly connected with the trend of the market itself.
In Italy, the restaurant and hospitality segment is the first in terms of number of new business open in the third quarter of 2019 (+0.98%) (source: Unioncamere, Infocamere) "Out-of-home"

consumption has progressively gained market share, thanks to a significant cultural and structural change that can support the sector in the long run (Figure 2).

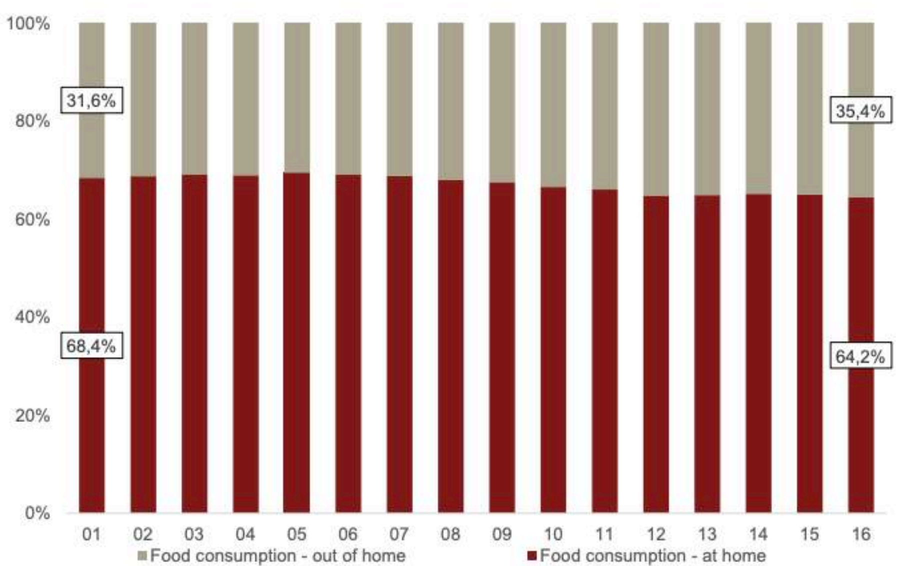


Fig. 2. Food consumption at home and "Out-of-Home" (2001-2016, share % constant prices). Source: ISTAT – FIPE, "The commercial food service market in Italy" report, PWC analysis 2018.

In the American market, one of the most important country for the vinegar sector and for company's business, the leisure and hospitality industry—which includes restaurants as well as accommodations and recreation/entertainment—has grown as a share of employment consistently since 1950 (Figure 3).

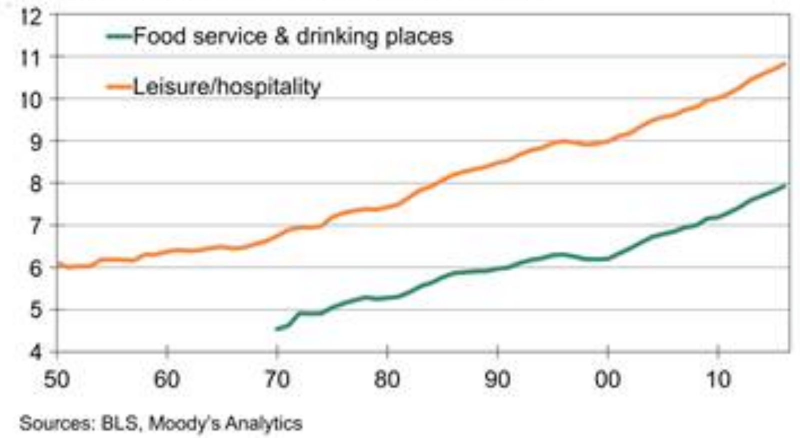


Fig. 3. Leisure/hospitality grows in the long run (industry share of total nonfarm employment, %)

Out-of-Home is become a very crowded and competitive segment and differentiate the service and products offered, rather than simply lower the price, is one of the key for the success. Differentiation in service and products is one of the drivers that impacted the growth of Out-of-Home sector, intended as the “request of higher quality and value food” from the customers. The latter is also one of the two most relevant trends in Food Service for the next future (and second only after the continuing growth of franchising trend) (source: “The commercial food service market in Italy” Report, PWC analysis 2018). In order to cope with the erosion of margins and the decrease in flows (e.g. competition with other forms of food service), small operators who have not converted into chains have focused their gastronomic proposal on high quality and healthy food to meet the increasing targeted needs of customers (e.g. organic food, vegetarian options, product recovery and artisanal processing methods).

The transition from a cheap product to a premium product (only if able to convey higher perceived value) is also another driver to differentiate the offer, in an asymmetrical approach to the competitive scenario, made up of multi-fragmented small businesses, towards new product with higher positioning.

The innovation in this sector must face with the challenge of transfer a higher “perceived quality” of the new products.

1.2.4 Acetaia San Giacomo Company market positioning

The company Acetaia San Giacomo mainly addresses the Food Service market with a larger quota (>80%) exported. In recent times, the company, which produces both PDO Traditional Balsamic Vinegar, certified organic Balsamic Condiments and certified organic vinegars made only with surface fermentation, has seen a growth in selling to restaurants with an average yearly increase of 8% (in the last 5 years). The food-service sector count for 74% of the business (in 2018).

Acetaia San Giacomo has a medium-high market positioning, gained after 20 years of business activity with "premium price" products.

The company initially offered his products to both channel, retail and food service.

The latter channel must be intended as “specialty food retail” (small independent store for European market, larger store but satisfying niche and luxury segment for international market). This segment is not monitored with regards of vinegar consumption and is not possible to perform a figure based assessment. However, according to company’s experience in this sector and despite the need of luxury and gourmet retail store to increase the offer differentiating from the mass

market, higher price remains an important threshold. Instead of conveying added implicit value due to different and less industrial production method, all the game is playing around a different and more attractive packaging, less minimal compared to the same product sold in larger retail store.

In the last 10 years, the company refocused on supplying more Out of Home sector, directly in Italy or through Food distributors specialized in food service, outside Italy.

Larger size and format of product available (when possible, due to mandatory size and shape of bottle for PDO Traditional Balsamic Vinegar) for Artisanal Balsamic or for wine vinegars, has been the key for a lower impact on price for the food cost in food service.

As it seems emerging from private consumption, which is growing but changing, looking for different and new vinegar-based product (also drink and beverage), also food service sector is evolving in vinegar consumption. There will be a massive demand for vinegar as “commodity” (the aforementioned growing trends for vinegar consumption due to its application as staple condiment common to many food cultures) where price and value added is close to zero.

However, the higher is the Out-of-Home consumption, the more differentiated and segmented become. This complex sector is evolving, through a highly-customized offer, created directly in the restaurant kitchen or as a demand for more and more customized product to the manufactures.

Technology, once applied only to industries, is now adapting to productions on ever-small scales, for private individuals or restaurateurs.

1.3 New market trends and new opportunity due to emergent needs

The market assessment reveals growing demand brought by the upcoming trends in food consumption. The aim of the analysis is to correlate the assessed trends and needs with possible solution in the “vinegar sector” with a specific attention to micro and small businesses.

1.3.1 “Prosumption”, Do It Yourself and Open-innovation

Over the last few centuries, the economy of the developed world has been dominated by the production/consumption paradigm. Its early years were dominated by production, especially in the factory. Much more recently, the focus shifted to consumption (with the shopping mall coming to rival, or even supplant, the factory as the center of the economy).

Prosumption (the term "prosumer" is generally attributed to Alvin Toffler, 1980) involves both production and consumption rather than focusing on either one (production) or the other (consumption).

Since the First Industrial Revolution, consumers have been mainly considered as playing a passive role with regard to production. However, the recent decades have seen a progressive growth in consumer involvement in production processes, for instance, in the form of user innovation, DIY and mass customization. Yet, it was not until the advent of digital technologies that consumers' input in production processes could become really significant in all dimensions of production (design, manufacturing and distribution). This increased role of consumers in production has been referred to as 'prosumption'. While prosumption has so far been mainly significant online (where consumers have arguably taken over the creation and distribution of content), recent advances in digital technologies (mobile networks, 3D printing, Internet Of Things, small scale and affordable equipments) have enabled prosumption to reach to world of physical objects, as illustrated by the increased importance of consumer-made goods ('makers' movement) and of the 'sharing economy'.

Given the recent explosion of user-generated content online, and an increasing call to action for the consumers into the chain of creation of the value (value co-creation and Wikinomics; Tapscott and Williams, 2006), we have reason to see prosumption as increasingly central.

The tendency interacts with a continuous segmentation of the market. Mass production doesn't fit the needs of current consumers in having more customized product.

This approach will lead the companies at least to change their approach from a "product first" to a "market first", then to open their decisional process to a new model of Open-innovation were stimulus and part of the value is directly created by the consumer.

Prosumption in the food sector can be approached looking at different trend started years ago based on the production of fermented foods.

This trend is growing in small high-end restaurant and for private consumers.

Freeman and Hannan (1983), detailing the importance of market niche in organizational ecology, focused on the restaurant industry. Restaurants are competitive small businesses in a segmented environment. In this free market, product differentiation is crucial.

The amount of the affordable technology now available, allow the restaurateur to became more "producer", dealing with the companies and became part of the production process.

Companies soon must face the opportunity to supply basic raw materials instead of standardized final product. Part of this research was focused on supply them the knowledge and the technology necessary to develop a self-production habits for not professional user. Private consumer is already experiencing this approach thanks to innovative systems now available.
With regards of vinegar production, the new and more active role of the consumers in the production process, can have different effects:

- Private: are willing to produce by themselves (DIY) the food and the ingredients they use. In the recent year, different machine or Kit for home-production of bread, beer, micro-green, jam, honey, etc became popular. The company could supply the kit, the procedures at the beginning and keep supplying the ingredients and consultancy.

- Food Service (Restaurants, Chefs): as for the private consumer, even restaurateurs (and small-business's owner like agritourism) they can wish to self-produce vinegars or, having a greater capacity of consumption, they can interact with the producer, with the company, in order to decide the characteristics of a customized product.

1.3.2 Circular Economy

The main aim of the circular economy is considered to be economic prosperity, followed by environmental quality. In comparison with "sustainability", differences and conceptual contour are blurry even if they are strictly integrated. The circular economy is now considered as a promising approach to help reduce our global sustainability pressures.
The execution of a Circular Economy approach, require effort at many levels such as implementation of practices of Re-use, Re-cycle and Zero waste. These approaches are gaining traction into the population and a lot of actions, private and public, are taking place in this direction.
During the research, we investigate if acetic fermentation can also play a role in the aforementioned context:

- use the acetic fermentation to transform leftovers from food processing;
- allow consumers in making their preferred vinegars using leftover wine, spirits, beer or food scraps;

- use vinegars as preserving liquid reducing waste;
- use food scraps or leftover food or wine as preferred carbon source for production of bacterial cellulose by specific strain of Acid Acetic Bacteria (AAB).

1.3.3 Health-conscious consumers

Recently, the focus of scientific investigations has moved from the primary role of food as the source of energy and body-forming substances to the subtler action of biologically active food components on human health. There has been an explosion of consumer interest in the active role of food in the well-being and life prolongation, as well as in the prevention of initiation, promotion, and development of non-transmissible chronic diseases. As a result, a new term - functional food - was proposed (Granato et al., 2010).

The rise of functional foods has occurred at the convergence of several critical factors, such as: awareness of personal health deterioration, led by busy lifestyles with poor choices of convenience foods and insufficient exercise; increased incidence of self-medication; increased level of information from health authorities and media on nutrition and the link between diet and health; scientific developments in nutrition research; and a crowded and competitive food market, characterized by pressurized margins (Siró et al., 2008). These factors have created a dynamic functional food and beverage market, offering good prospects for growth for well-positioned food and drink manufacturers. Between 1998 and 2003, global value sales increased by almost 60%, and by almost 40% in 2008 (Euromonitor 2009).

These may be defined as foods and food components that provide a health benefit beyond basic nutrition (quantities necessary for normal growth and development) and include conventional foods, fortified, enriched or enhanced foods and dietary supplements (Clydesdale 2004).

Functional foods and natural health products are a relatively new component of the human diet with important policy implications. Functional food refers to food that is intended to be consumed as part of a normal diet and contains ingredients that have the potential to enhance human health or reduce the risk of disease beyond basic nutritional functions. Natural health products (NHPs), also known as nutraceuticals or food supplements or nutritional supplements, are products that have been isolated or purified from food and may include ingredients such as amino acids or vitamins; they tend to be marketed in the form of pills, powders, capsules or tablets (Malla and Hobbs, 2013).

Consumers are more conscious of the maintenance of good health through diet, thereby gravitating towards foods that offer additional benefits beyond that provided by conventional food, and are demonstrating a willingness to pay a premium price for these products.
New R&D applications that promise growth opportunities in the food industry are of interest from an agricultural and food policy perspective.
With regards on vinegars, their functional properties and their bioactive compounds (Ho et al., 2017) can play a strategic role if used as condiment or as base of an innovative drink. The challenge of this research was to investigate whether a different approach than a mass production, in favor more customized product, was implementable.

1.4 Regulatory context analysis

In 2016, the classification of the Vinegar sector has been modified through European and Italian law's amendment.
The European Commission published Commission Regulation (EU) 2016/263 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and Council as regards the title of the main food category "12.3 Vinegars". The new title of the food category 12.3 is now: Vinegars and diluted acetic acid (diluted with water to 4-30 % by volume). The main category was renamed because in some Member States only vinegars obtained from the fermentation of agricultural products are allowed to be named 'vinegars'. In other Member States, however, both products obtained from the dilution with water of acetic acid and vinegars obtained from the fermentation of agricultural products are marketed under the name 'vinegar'.

	EU FOOD CATEGORY	
12.3	Category: Vinegar	
	Food grade Acetic Acid diluted with water	Vinegars from fermentation of agricultural products

The Italian wine sector has been also recently modified through a comprehensive reform and re-organization by the law n 238, December 12, 2016 know as "Consolidated code for Organic discipline of vine cultivation, wine production and wine trade".

This reform also interested the “Vinegars sector” and de-facto recognized, for the first time, a “new” sub-category of vinegars. In this context “novelty” must be intended as identification, for the first time, of certain products (vinegars) to whom, because of certain characteristics regarding the production method, a special treatment is applied in the food regulation.

The Consolidated code, at the Title V (Vinegar’s discipline) article 49, paragraph 2, reports:

“*By way of derogation from paragraph 1, wine vinegar is the defined product by the current European Union legislation containing a quantity of ethyl alcohol not exceeding 1.5 per cent by volume. For the wine vinegars obtained by static fermentation and maturation in wooden containers, or even of different material for vinegars whites only, with a capacity not exceeding 10 hectoliters for a period not less than six months, the aforementioned limit of 1.5 per cent by volume it is raised to 4 percent by volume*.”

<table>
<tr><td></td><td colspan="3">EU FOOD CATEGORY</td></tr>
<tr><td>12.3</td><td colspan="3">Category: Vinegar</td></tr>
<tr><td></td><td>Food grade Acetic Acid diluted with water</td><td colspan="2">Vinegars from fermentation of agricultural products</td></tr>
<tr><td></td><td colspan="3">ITALY</td></tr>
<tr><td colspan="2"></td><td colspan="2">Vinegars from fermentation of agricultural products</td></tr>
<tr><td colspan="3">→</td><td>A new sub-category for wine vinegar identified</td></tr>
</table>

In the EU, Regulation (EC) 1493/1999, there are currently established thresholds for acidity and residual alcohol. Hence vinegars are those products having a minimum 5 % (w/v) acidity and a maximum of 0.5 % (v/v) ethanol, with the exception of wine vinegar which is exclusively obtained from wine and whose acidity is 6 % (w/v) at least and has a maximum ethanol concentration of 1.5 % (v/v).

This exemption regarding the maximum ethanol concentration, from 1.5% to 4% (v/v), is justified by the production processes used by small factories where, following a surface fermentation and not diluting at the beginning nor at the end of the process, the ethanol content of the final product can be greater than 1.5%. Through the exemption, the Parliament acknowledged a production niche represented by small artisanal producer.

This derogation, followed the Protected Denomination Origin rule of Sherry vinegar (Vinagre de Jerez DOP, according to EU official journal, 2011) which has 2 different exemptions for the maximum ethanol content. Up to 3% in volume for the regular version and up to 4% in volume for Pedro Ximénez and Moscatel vinegars.

The company contributed to this modification, supplying from the 2014 and 2016, data and documentation to the XIII Agricultural Commission during the preparatory works of the law's project.

We here point out that, during the preparatory works, the practice of the "dilution with water" of the vinegars prior and/or after the acetic fermentation, was also discussed.

1.4.1. Defining the identified sub-category of vinegars

1.4.1.1 New wine vinegar sub-category as valorization of production niche

The aforementioned reform acknowledged the request that came from a production niche that needed different characteristics for the go to market of their products. Without this exemption, vinegars made with static superficial fermentation (and without water dilution), they could not fit into the legal parameters.

Is not the first time that the Legislator acknowledge and recognize, giving a regulatory protection, a demand from small-medium manufacturers.

The perfect example is given by the "Artisanal Beer".

As for the total beer production in Europe, in 2016 it is estimated that it has reached 400.2 million hectoliters. These production levels lead Europe to be the second largest world producer, immediately after China (497.8 mln hectoliters), far exceeding the productions of States United States (226 million hectoliters) and Brazil (140 million hectoliters) (FAOSTAT, 2014).

Italy is the only country in the world to have adopted a legal definition of "Artisanal Beer". The adjective has been considered as an indispensable tool of communication towards consumers and able to convey a strong product identity.

The new consolidated code however, didn’t give a specific name to that sub-category of vinegars.

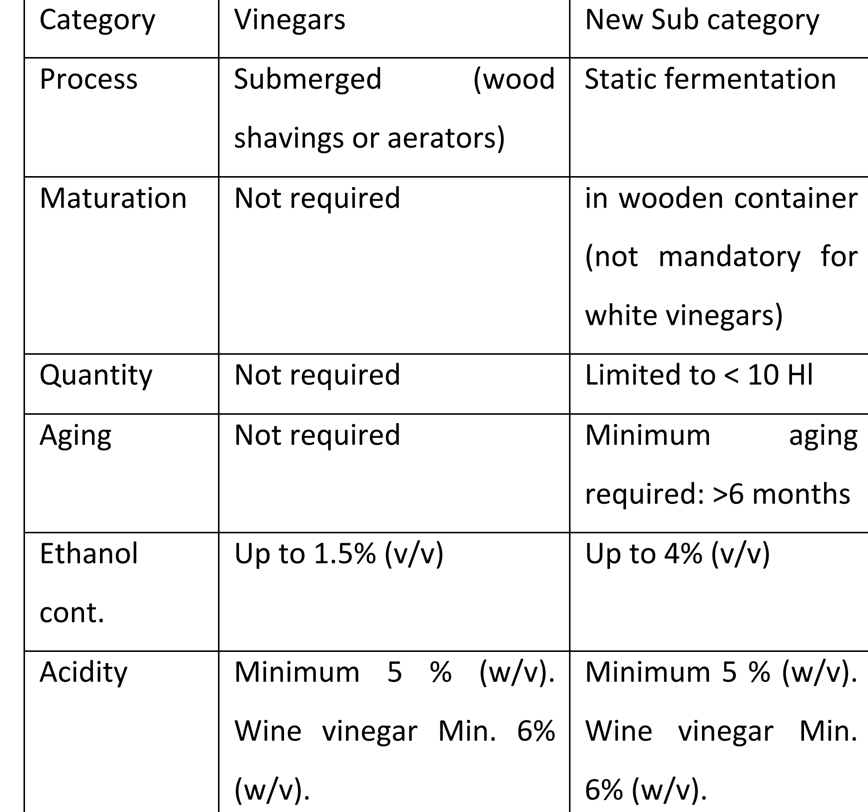
The definition “*Wine vinegars prepared with artisanal methods, with long maturation*” was stated in the first drafts of the law’s proposal. That definition has been removed after the last amendments proposed by Federvini (Italian Federation of Industrial Producers, Exporters and Importers of Wines, Sparkling Wines, Aperitifs, Spirits, Syrups, Vinegar). Simultaneously, the limited quantity, the wooden container for maturation and the minimum aging were introduced.

Based on the processing technology employed, vinegar production can be broadly divided into two categories, surface fermentation and submerged fermentation processes (see chapter 2).

In literature, the two categories of vinegars are usually indicated referring to the method/process used: “Traditional” and “Industrial” (Ebner et al., 1996; Gullo and Giudici, 2008).

In surface fermentation, also called the static method, the AAB grow on the surface in contact with atmospheric oxygen. Therefore, it requires a long time to yield the final product, vinegar. In contrast, the submerged fermentation process involves direct injection of oxygen into the substrate liquid using a generator or an acetator to increase the surface area, and therefore, the fermentation is faster. The surface fermentation method is employed to produce traditional and selected vinegar, despite disadvantages such as a long fermentation period, a risk of contamination, and difficulty of quality control. This is not only because volatile aromatic compounds are retained, but also because the initial investment is more economical than that for the submerged fermentation process

Due to the identified production characteristics, the vinegars mentioned in the law’s reformation, are produced in a way that is different to those included in the standard category. Others characteristics are also required by the law such as, a maturation in wooden container, a limited quantity for each batch and a minimum aging required (Table 2).



Category	Vinegars	New Sub category
Process	Submerged (wood shavings or aerators)	Static fermentation
Maturation	Not required	in wooden container (not mandatory for white vinegars)
Quantity	Not required	Limited to < 10 Hl
Aging	Not required	Minimum aging required: >6 months
Ethanol cont.	Up to 1.5% (v/v)	Up to 4% (v/v)
Acidity	Minimum 5 % (w/v). Wine vinegar Min. 6% (w/v).	Minimum 5 % (w/v). Wine vinegar Min. 6% (w/v).

Table 2. Differences between “Traditional” and “Industrial” method/process.

Only the “method”, is the feature that could be use as driver to investigate the possible name to that sub-category of wine vinegars. The surface fermentation method has been always indicated as “Traditional method” but, with the indication of a new sub-category, it seems that definition is not appropriate anymore.

1.4.1.2 Traditional

By analyzing the regulatory European and Italian context, for the identified category, adjectives such as "Traditional" or "Artisanal" could be equally used in defining the new sub-category.

In 1992, the EU introduced a system of protection of geographical indications similar to the system already available in France

The objective of Regulation (EEC) No. 2081/92 is the protection of geographical indications as names for food products. The aim of Regulation (EEC) No. 2082/92 is the protection of traditional recipes for food products, and the objective of Regulation (EEC) No. 2092/91 is to explicitly define the objectives, principles and rules applicable to organic production. Products protected by these

EU quality schemes have a privileged position, not only with respect to legal protection, but also with respect to EU financial aid and the possibility of member state financial aid for their promotion.

Three EU schemes of geographical indications and traditional specialties, known as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialities Guaranteed (TSG), promote and protect names of quality agricultural products and foodstuffs. Products registered under one of the three schemes may be marked with the logo for that scheme to help identify those products. The schemes are based on the legal framework provided by EU Regulation No 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs. This regulation (enforced within the EU and being gradually expanded internationally via bilateral agreements between the EU and non-EU countries) ensures that only products that originate from that particular region are allowed to be marketed as such. Regarding vinegars, there are currently five PDO registered categories and one PGI. Among PDOs:

- three from Spain: Vinagre de Jerez, Vinagre de MontillaMoriles, Vinagre de El Condado de Huelva
- two from Italy: Aceto Balsamico Tradizionale di Modena (TBVM), Aceto Balsamico Tradizionale di Reggio Emilia (TBVRE).
- Lastly, Aceto Balsamico di Modena is registered as a PGI (BVM)

The "traditional" definition used in the Geographical Indication framework, however:

1- is already used in 2 PDO vinegars (TBVM and TBVRE),
2- seems to be too broad and possible source of confusion.

In fact, in Regulation (EEC) No. 2082/92, the Article 4 reports:

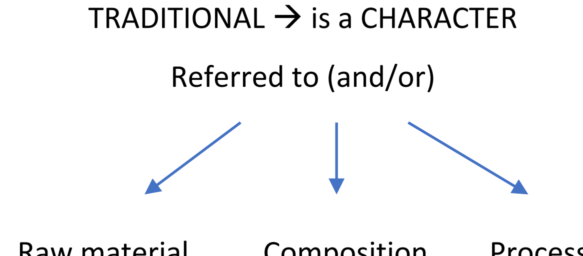
In order to appear in the register referred to in Article 3[1]*, an agricultural product or foodstuff must either be produced using traditional raw materials or be characterized by a traditional composition*

[1] Article 3 The Commission shall set up and administer a register of certificates of specific character which will list the names of agricultural products and foodstuffs of which the specific character has been recognized at Community level in accordance with this Regulation.

or a mode of production and/or processing reflecting a traditional type of production and/or processing.

TRADITIONAL → is a CHARACTER

Referred to (and/or)



Raw material Composition Process

Also, in EU Regulation No 1151/2012, the article 3, sets the “Definitions” that shall be applied for the purpose of the Regulation. Defining the term “Traditional”:

- *means proven usage on the domestic market for a period that allows transmission between generations; this period is to be at least 30 years;*

TRADITIONAL → is the PROVEN USAGE

We assessed that the term "traditional" is used in the European regulatory context, more like a "historical" character of belonging to the long culinary tradition and refers to a “Country-of-origin”, typical of Mediterranean countries. Traditions or characteristic linked to geographical origin and not, or not only, with unique reference to the production method.

Also, the PGI Balsamic Vinegar of Modena, considered Traditional products, contains not less than 20% of concentrated and/or cooked grape’s must and not less than 10% of vinegar to whom mostly submerged acetic fermentation is applied. This is an interesting example because despite the industrial production (98.000.000 of liters per year) it can be technically considered “a traditional product”.

For these reasons, and also because the word “Traditional” is already used for two vinegar’s Geographical Indications, we have not identified as "traditional vinegars" the sub-category identified by the reformed law, because lack in distinction and source of potential confusion.

1.4.1.3 Artisanal

In general, according to Italian legislation, a product can be defined as "artisanal", if produced by a company that has the requirements to be recognized as "artisan factory" (Impresa Artigiana) following the current legislation.

According to the Consolidated Code 443 August 8th, 1985 (subsequently modified in part by the Act 57 March 5th 2001), it is an artisan factory that falls within dimensional limits (considered in number of employees, different depending on the sector in which it operates) and if, from the productive point of view, it has a prevalence of the human factor with respect to the capital / technology used. The "lower technology" involved must be considered not necessary with reference to the process but, in general, as a prevalence of the human work compared to the equipment as company assets. A high labor-to-capital ratio business.

In the food and wine sector, only beer has obtained a specific definition and consequently a law, for the "Artisanal" category.

In 2016 the definition and relative discipline of "Artisanal Beer" has been introduced by the law n. 154 of july 28th 2016.

According to article 2nd paragraph 4bis, is "artisanal" the beer that

- is produced by Independent and micro-brewery
- is not pasteurized nor micro-filtered

Other application in food category of the adjective "Artisanal" (Craft) seems to be on their way to be recognize. The "artisanal ice cream" for instance, seems to be recognized as standing alone category through two legislative proposals submitted in 2016[2] and 2017[3]. Analyzing these two proposals, the "artisanal" character refers to the process and to the raw materials and was used to describe the "High quality ice cream" (Gelato artigianale di alta qualità).

At European level, the definition of "artisan company" is not exploited in company's classifications, which instead defines the micro-enterprise, identifying for this purpose size limits of employees and annual turnover.

However European legislation is evolving in the direction of guaranteeing the quality of food products.

This action is taking place recognizing the difference in production methods that ensure the respect of "traditional" or "artisanal" product.

The EU Geographical Indication (PDO, PGI, STG) ecosystem, as previously said, is one of the most complex with regards of protection of "Traditional" food and wine products. These "traditional products" can be processed with "Traditional method" or with an industrial approach.

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[2] Proposal n. 4181 for a parliamentary initiative, entitled "Provisions concerning the production of high quality artisan ice cream", was presented on 21 December 2016.

[3] Proposal n. 4236 with following title: "Introduction of the denomination of " artisan gelato of Italian tradition "and provisions concerning its production". Presented on 18 January 2017.

The aforementioned laws, considered the basis of the whole quality food schemes, never use the term "artisanal" in their statutes.

Only recently the EU Commission used for the first time the word "Artisanal" in declaring the first celebration day for a food product[4] (artisanal ice cream) and conceiving that adjective more related to the traditional method plus other implicit features typically found in the artisanal process (fresh raw materials, no additive nor artificial aromas).

In establishing the European Artisanal Gelato Day, the European Parliament defined the agri-food product as "excellence in terms of food quality and safety". Beside the concept of "safety" which must be considered as a pre-condition, again the "craftsmanship", the "artisanal" feature, is strictly linked to "high quality product" idea.

According to the Unesco definition also:

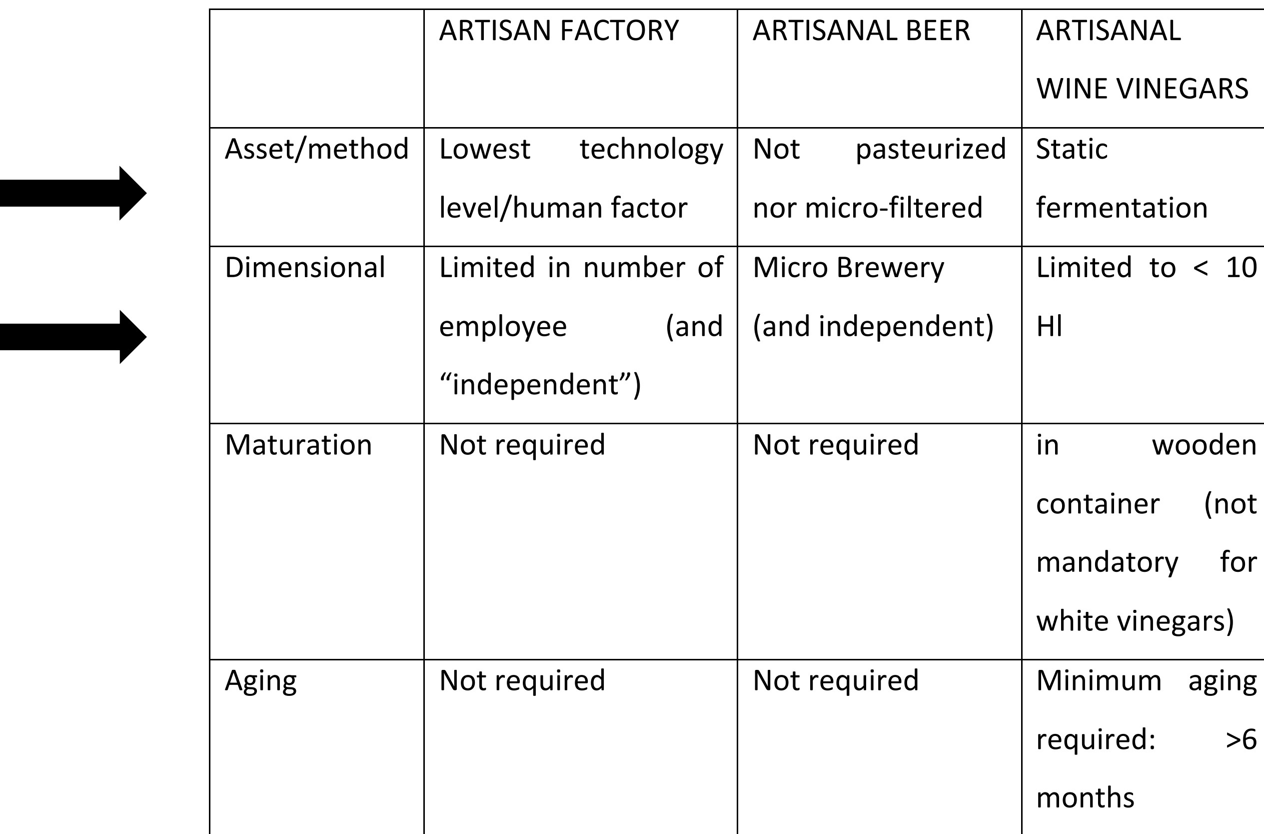
Products that are produced by artisans, either completely by hand or with the help of hand-tools or even mechanical means, as long as the direct manual contribution of the artisan remains the most substantial component of the finished product… The special nature of artisanal products derives from their distinctive features, which can be utilitarian, aesthetic, artistic, creative, culturally attached, decorative, functional, traditional, religiously and socially symbolic and significant".

As a conclusion, the definition "artisanal wine vinegars" used in this research, complies with Italian and European legislation and is capable of accurately characterizing the recently identified sub-category.

By this definition, we mean the coexistence of at least two conditions: made following a traditional method with high labor-to-capital ratio manufacturing, and limited quantity as implicit characteristics of the product (and considering the normative and food-safety as pre-conditions).

We also concluded that the adjective "artisanal" can be adopted if the identified conditions (Table 3) are respected.

[4] in 2014 the European Parliament established the European "artisan gelato day", to be celebrated on March 24th, to contribute to the promotion of this product and to the development of the gastronomic tradition of this sector.



	ARTISAN FACTORY	ARTISANAL BEER	ARTISANAL WINE VINEGARS
Asset/method	Lowest technology level/human factor	Not pasteurized nor micro-filtered	Static fermentation
Dimensional	Limited in number of employee (and “independent”)	Micro Brewery (and independent)	Limited to < 10 Hl
Maturation	Not required	Not required	in wooden container (not mandatory for white vinegars)
Aging	Not required	Not required	Minimum aging required: >6 months

Table 3. Characteristics of artisanal method and products.

1.5 Artisanal claim as new added value

The examination aimed at giving a definition goes beyond the merely classifying will.

This investigation try to define the features that vinegars category can have in order to gain added value, not only in a functional meaning.

The added value gain more importance for higher level of market segment (niche and luxury segment).

And it seems that also on a lower market segment (mass- and mid-market) is becoming a valuable claim.

Recently, Jury of the Institute of advertising self-discipline (rules n. 56 october 25th, 2019) has censored the TV commercials and the wordings on the packaging of "Le Artigianali" made by a big industrial corporate. According to the Jury, the use of the term "artisans" referring to products is misleading, and therefore contrary to Article 2 of the Advertising Self-Regulation Code.

According to the Jury the expression "craft" has a clear and precise meaning. It is not just a question of simplicity and genuineness, but above all of a "processing method that contrasts with the whole industry, referring to techniques prevalent in traditional sectors, non-automated processing in some form of intervention, including manual intervention"

We could assess that “artisanality” (craftmanship) is so fare the only feature that could differentiate an industrial product from another made, precisely, in a slower, smaller and with traditional method. Indubitably for some consumer’s clusters, this is an objective added value, no matter about the organoleptic quality or other official certification (organic, health claim, country-of-origin).

The values connected to the identified category are to be ascribed to another domain, different from the functional, health, dietary or even mere sensorial quality properties. They can be classified as hedonistic properties.

Vinegars already has a recognized basis of nutritional and functional values Functional Properties of Vinegar (Budak et al., 2014), we tried to go beyond the added values thus considered, both because they are not yet sufficiently detailed in the literature, as well as sometimes discordant, even because, with specific reference to sensorial quality, of dubious effectiveness at the time of go-to-market.

It has been pointed out how the consumer can perceive as healthier a product with a "healthy" claim or as less fattening if the claim is dietetic.

Or perceive as "sustainable" and of “low environmental impact” (green) for the certified organic product, they are dynamics that fall within the complex relationship between claim, actual effects and impact on the purchase motivation.

Or again how the product that meets the requirements of the PDO PGI STG European schemes can be perceived as "historical, linked to a specific area of origin". In this case, the motivation is not functional, but it is probably a mix between a "guaranteed quality" (although this concept is too generic), a product that is made: according to traditional criteria with selected raw materials, which follows historical production methods. However, it is believed that these values are mainly of a hedonistic nature.

Even the claim "Artisanal" (with the set of values capable of conveying) could have an effect that has repercussions on different expectations, classified as hedonistic. We have in fact assessed that at the level of Italian and European legislation, the term artisanal was intended as for a high-

quality product. Although the concept of "high-quality" can be generic, it is believed that in the regulatory context, the tendency is to favor the positive meaning of the term "craft", “artisanal”.
The values that this term can convey and therefore add to the food safety regulatory preconditions, can be ascribed, at a minimum level, to a product made:
- with traditional methods
- available in small quantities

It is believed that this is the set of minimum positive values that the concept of “artisanal”, in general or applied to a product as a claim, may be able to transfer as an added value. This set of values can in turn be combined in the concept "(Greater) quality" or at least “Done with care” and also more exclusive, unique product.
An artisanal food item can also be conceived as being a cultural product (Tregear, 2003; Van der Meulen, 2007).
This distinctiveness brought by the term in exams, presents value-adding potentialities and regional development opportunities (Marsden, Banks and Bristow, 2000a; Gralton and Vanclay, 2006; OECD, 2006)
In this sense, craftsmanship, limited production, exclusivity are elements of great importance for this market segment.
Artisanality in relation to food processing refers to a high labor-to-capital ratio in the transformation of raw agricultural product into a final food item (de Roest, 2000). This high labor input adds value to the raw agricultural product with artisanal products selling for many times the price of non-artisanal equivalents (Marsden, 2003). Artisanal food creation sits in stark contrast to the industrial agri-food system with its highly mechanized, highly processed, mass produced and standardized food production.

1.6 Innovation

According to general guidelines for measuring innovation as defined in the Oslo Manual (OECD, 2005):
An innovation is the implementation of a new or significantly improved product (good or service), or process, a new marketing method, or a new organizational method in business practices, workplace organization or external relations.
The definition of innovation take also in exam the attitude of disrupt an existing technology or not, depending the level of novelty introduced.

In this research, we applied a concept of incremental innovation rather than radical innovation (disruptive). If radical innovation corresponds to a fundamental change that is expressed in revolutionary modifications of product / process. An incremental innovation, on the other hand, is an improvement or adjustment of existing product / process.

Another definition is also important for the purpose of this research. Hirschman (1981) introduced the concept of "symbolic innovation" where is not only the technological, functional or physical change meets a need for a superior performance, but also the innovation that brings new social meanings and value (perceived value).

The boundaries of this field are blurry and scientifically difficult to define.

The question is if the "Artisanal" adjective and production method, beyond functional and technological added value, could be also considered a driver to add "symbolic innovation", increasing the value.

The company aimed to seek an innovative approach to a production always considered "traditional" where, instead, new and innovative products are gaining more and more important market shares.

The Acetaia San Giacomo company fits in the description of the typical micro-business that, together with the small and medium company, represents more than 94% of the Italian economic scenario. These kinds of company invest in R&D the 0.17% of the annual turnover (Banca IFIS; Business Register, 2018).

The designed matrix (market and competitive analysis), aims to be a model for micro and small business, we have assessed an empty space for innovative application. That space is reachable towards the combined adoption of 2 incremental innovation drivers.

1. Technological innovation, in response of emerging needs, can impact
    a. the operator and/or environment
    b. the raw material adopted
2. Symbolic innovation, in order to reach higher market positioning, can impact
    a. the method, leveraging the way *how* the biochemical process of fermentation is conduced.

1.7 Conclusion

The conceptual framework definition aimed to assess the state of the art of the vinegar sector, trying to identify possible space of innovation in a segment usually considered “Traditional” and “cheap” (vinegar as commodity): the latter market-valorization reflects the general perception of vinegars as staple and basic ingredient or “leftover” or a good raw material, gone bad (generally wine or beer).

However, all the insights emerging from the market seems to indicate good opportunities to take advantage of an open, wider perspective from a new generation of consumers.

Crossing actual data and figures, trends and market segmentation, Company’s case history, is possible to design the conceptual framework with a go-to-market approach.

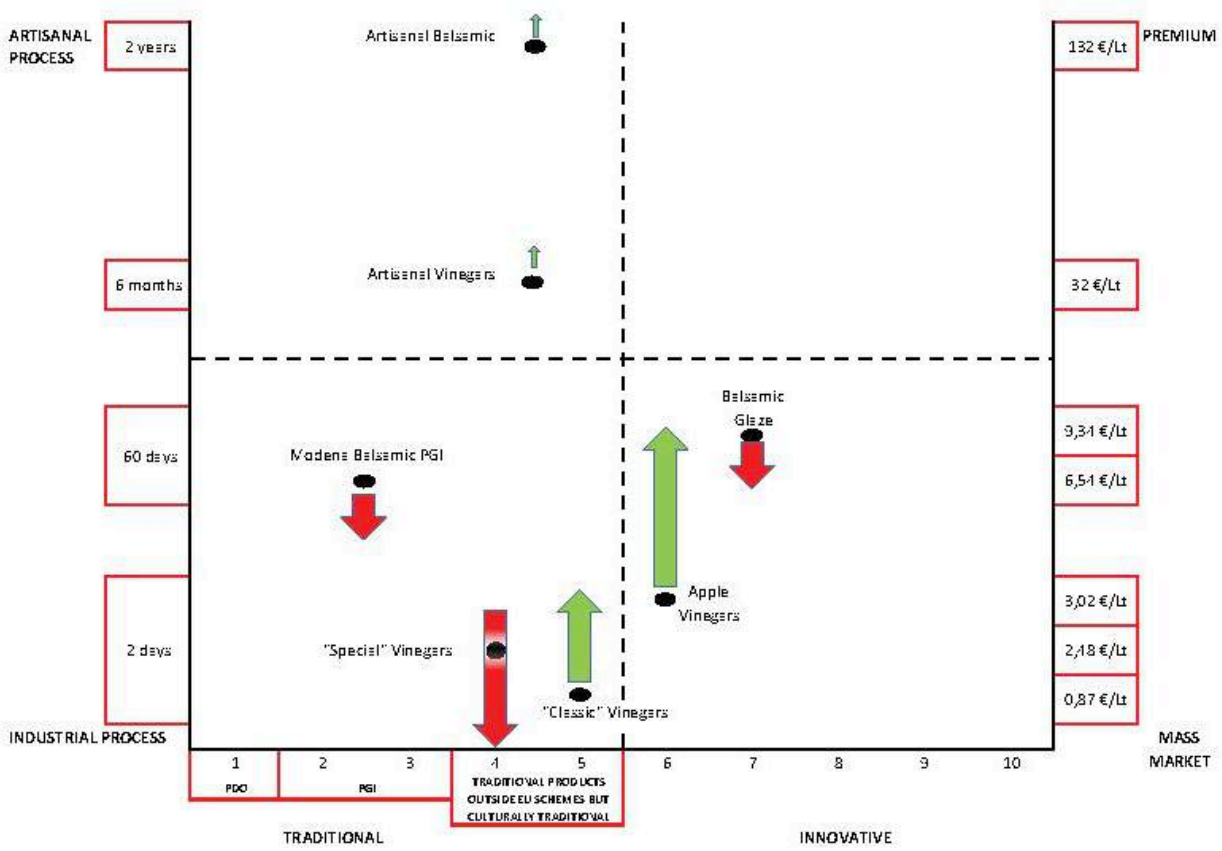


Fig. 4. Market, competitive and productive analysis matrix.

In Figure 4, the left vertical axis represents different degrees of "Industrialization" of the process. The objective parameters for this adopted for this kind of evaluation, refer to the legal requirement for the minimum aging. We assumed that, according to the definition of the new sub-category of wine vinegars, higher is the minimum aging, more "artisanal" is the process adopted.

The right vertical axis is figured out using the average price per Liter (Euro, Italian retail price included VAT) according to the market analysis and company's data. This axis is also strictly connected with market segmentation: mass market at the bottom, mid-market and then "premium" or "niche".

The horizontal axis represents the scale of Traditional/innovative value. The objectivity of "Traditional" products is given by the recognition of the EU schemes, decreasing from PDO to PGI, then to "traditionally and historically well-known and produced". Is undoubted that wine vinegars ("classic" or "special") have been produced for years. "Balsamic Glaze" has a difficult positioning in the matrix because it technically a newer product but, at the same time, most of the time has a

PGI Balsamic Vinegar of Modena as main ingredient that lead the perception as a "traditional" product.

The red and green arrows represent, the sale's trend. Green stands for growth, red for a decreasing trend. The size of the arrows is proportionally related to the entity of the trend (Table 1).

In the upper left quadrant, the company's product positioning according to the correlated factors.

Traditional Balsamic vinegar of Reggio Emilia PDO are not represented in this matrix. With its minimum 12 years of aging required by the quality scheme, and an average price per Liter of approximately 1.000 Euro, it perfectly fits in the "Luxury" segment which is the highest after "Niche".

As result we can notice in Figure 5 the upper right quadrant as a possible space for the incremental innovation application.

Considering the medium-high positioning of the company (mid-market, niche, luxury) and the company productive assets (artisanal company with low tech involved, the acetic fermentation are executed only with surface method), empty space for innovation is found on the artisanal-premium price vinegars (upper right quandrant).

Premium product needs a greater quota of added value brought by the innovation.

In order to reach that space, we assess that 2 innovations driver are required (Fig. 5)

One, the horizontal, applied to objective properties of the final product.

In this case, the output should meet the new customer's need with new, functional or utilitarian features. The innovation should shift the product portfolio towards new way of use (drinks, small scale production), or new product adopting different and unconventional raw materials, different flavor profile.

The vertical arrow represents the internal (to the company) innovation which is given by adopting a specific fermentation method (static superficial fermentation with long maturation).

Thus, this biochemical process, applied to the internal production process, is able to carry the "artisanal claim" to the final output, creating an objective asymmetry in the competitive landscape and elevating the perceived value of the product; no matter other quality schemes or certification that can be adopted by other companies of any dimensions.

The combined effect of the two innovative drivers are theoretically able to move the market positioning of the new product, in the empty space previously identified.

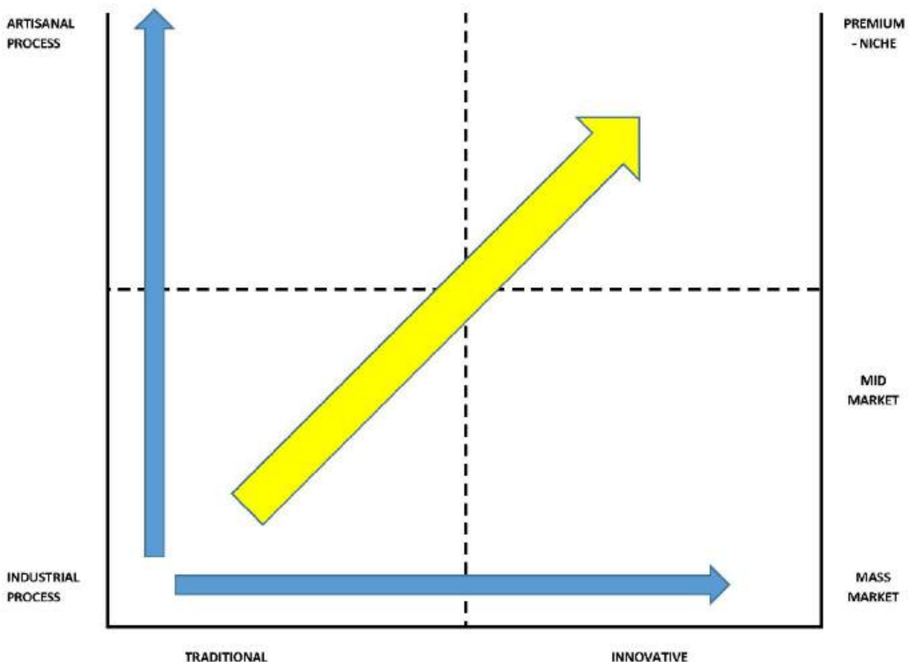


Fig. 5. Innovative driver application to vinegar market.

To conclude, the approach adopted in this research is based on these premises, performing an horizontal investigation about product and processes to whom apply the two innovative driver.

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Chapter 2

OVERVIEW ON BACTERIAL CULTURES PRODUCING ACETIC ACID, USAGE IN ACETIFICATION PROCESSES AND RELATED EFFECTS

CHAPTER 2 – OVERVIEW ON BACTERIAL CULTURES PRODUCING ACETIC ACID, USAGE IN ACETIFICATION PROCESSES AND RELATED EFFECTS

2.1 Introduction

Acetic Acid Bacteria (AAB) are the main organisms acting as catalyzing agents in acetic acid fermentation. The AAB group includes highly versatile organisms able to oxidize, under aerobic conditions, different carbon sources into compounds with different applications in food, beverages, chemical, medical, pharmaceutical and engineering fields (De Roos and De Vuyst 2018, Mamlouk and Gullo 2013).

Acetic Acid, as a result of the oxidative fermentation of ethanol brought by AAB (acetic fermentation) is the man organic acid present in vinegars as a solution. However, some identified species of AAB are also able to synthetize bacterial cellulose (BC) into the liquid culture, or metabolize polyalcohols, carbohydrates and organic acids into different compounds.

Despite vinegars is one of the most ancient fermented food, and the biochemical transformation is always the results of AAB activity, acetic fermentation processes are evolved and are nowadays different in terms of technology involved, efficiency, productive yield and time.

2.2 Acetic Acid Bacteria

2.2.1 General characteristics

Acetic acid bacteria, that belong to *Alphaproteobacteria*, are gram negative or gram-variable, non-spore forming, ellipsoidal to rod-shaped cells that con occur in single, pairs or in short chains. Catalase positive and oxidase negative, AAB have an obligate aerobic metabolism, with oxygen as the terminal electron acceptor. The optimum pH for the growth is 5-6.5 while they can grow at lower values between 3 and 4 (Sievers and Swings, 2005). Their optimum temperature vary between +28 and +30°C although some species are recently recognized as thermotolerant (Ndoye et al., 2006).

They occur in sugary and alcoholic, slightly acid niches such as fruits, flowers, honey and particularly beverages like beer, wine, cider, kombucha tea, vinegar and souring fruit juices. On these substrates, they oxidize sugars and alcohols into organic acids. When the substrate is EtOH, AcOH is produced, and is from where the generic name "acetic acid bacteria" of this bacterial group comes from. However, AAB are highly versatile microorganisms, in addition to their capability to oxidise EtOH in AcOH, it is reported as results as their metabolic activity, the possibility to oxidize different carbon sources like alcohols (primary and polyalcohols) into sugars, carbohydrates (sugar and sugars derivatives), organic acid; they are also able to fix free nitrogen into cell material by reducing it to ammonium (Diazotrophy) (Mamlouk and Gullo, 2013).
Recently, industrial interest focused on their metabolic ability on exopolysaccharides (such as dextrans, levans and cellulose) production.
This feature makes them valuable biocatalysts for a number of useful applications, but at the same time AAB are also spoiling organisms in some fermentation processes.
In addition, it is well known that a large fraction of microorganisms present in both natural and industrial environments are uncultivable under standard laboratory conditions. The uncultivability phenomenon limits the understanding of species richness and diversity and consequently a broad-spectrum strategy to select efficient strains as starter culture is affected. This phenomenon affects also AAB, for which the limit of culturable methods has been observed (Mamlouk et al., 2009).The difficulty of cultivating AAB is one of the reasons why vinegar fermentation is still performed using unselected cultures.

2.2.2 Classification

After their initial description as vinegar bacteria more than 150 years ago, AAB have undergone many taxonomic variations.

Acetobacter is the oldest genus; its description dates from early studies of vinegar microbiology (1898); the name defines 'vinegar bacteria'. In 1968, the genus *Gluconobacter* was introduced, based on the capacity to oxidize ethanol and glucose. The *Gluconobacter* species, which strongly oxidizes glucose and weakly oxidizes ethanol, did not oxidize acetate and lactate to carbonate and only exhibits polar flagellation if motile, were included in the new genus.

The genus *Acetobacter* included organisms that only exhibited peritrichous flagellation if motile, strongly oxidized ethanol, weakly oxidized glucose and completely oxidized acetate and lactate.

The genus *Gluconacetobacter* was established based on the differences in the ubiquinone (UQ) system. The species of *Acetobacter,* which were equipped with UQ-10, were transferred to this genus and the species with UQ-9 remained in the genus *Acetobacter*. In 2013, the genus *Gluconacetobacter* was separated into two phylogenetically and phenotypic groups, named as *Gluconacetobacter liquefaciens* and *Gluconacetobacter xylinus*. The latter group included species that were transferred to the new established genus *Komagataeibacter* (as reported in Mamlouk, 2012). Species of the genus *Komagataeibacter* have relevance to vinegar; for example, the species *Komagataeibacter europaeus*, that is highly tolerant to acetic acid and ethanol, is responsible for the production of high-acidity vinegars (Gullo et al. 2014). Now the group includes more than 18 genera, but species having biotechnological role are included mainly within *Acetobacter* and *Komagataeibacter* genera.

2.2.3 Optimal Temperature

AAB are mesophilic bacteria growing at optimal temperature of 28-30 °C. For values greater than 34 °C and less than 8 °C, the growth is generally inhibited. Otherwise, studies reported the capability of some thermotolerant AAB that are able to grow and to produce acetic acid in range of temperature values comprises between 36 and 45 °C(Mamlouk and Gullo, 2013). In submerged system, the optimal temperature for which a higher amount of acetic acid is produced were between 29 and 30 °C (Adams 1985).

In uncontrolled fermentation systems, like the production process of PDO Traditional Balsamic vinegar of Modena and Reggio Emilia (or other types of vinegar produced by static systems or artisanal manufacture), the growth of AAB and, consequently, the production of acetic acid, are completely dependent by the room temperature, causing variations of the fermentation process speed.

Furthermore, we should consider also that the ability to grow at different temperature is dependent from other parameters (acetic acid and ethanol contents, oxygen, pH). At the end, since the transformation of ethanol in to acetic acid is an exergonic reaction, the temperature could increase during the fermentation process. The variation and the increase of temperature during the summer season can affect drastically the fermentation process, when conducted without temperature control. In the present study, the temperature remains not controlled for all the process implemented at factory scale, causing short variations due to the environment conditions. However,

given the closed environment and artificial temperature changes, AAB should grow because the limiting temperature values that inhibit the AAB development should be not reached.

2.3 Acetic fermentation tecniques

From the manufacturing point of view, taxonomic issues do not have substantial relevance. Instead, the following phenotypic traits of AAB are very important in vinegar production:

• no exopolysaccharides production (thick layers of cellulose known as 'mother' of vinegar), which affects the yield and quality of the product. This trait is widespread among strains of the species *K. xylinus* and other closely related species;

• high tolerance to ethanol and the ability to grow in wines with more than 12 per cent ethanol.

Extrinsic conditions are also required in order to perform the fermentation process in the optimal way, like temperature, acetic acid content and oxygen dissolved into the media.
The correlation between the intrinsic attitude of the bacterial group as biocatalyst and extrinsic conditions, are the base of the technological evolution of fermentation process and method.

Vinegar brewing can be performed by two main systems. The first system is liquid fermentation, which comprises a set of techniques developed in Western and European countries. Among these techniques, the two main categories belong to the static superficial method and the submerged method
The second system is solid-state fermentation, which uses microorganisms grown on substrates in the absence of free water; this system is used to produce vinegar from grains in Asian countries. Solid state fermentation includes three main biological steps: starch liquefaction and saccharification, alcohol fermentation and acetic acid fermentation (Wu et al., 2009, Gullo et al., 2014).

2.3.1 Different methods in liquid fermentation system

Considering the raw materials used during this study, our focus is mainly on liquid fermentation.

Liquid fermentation system can be performed in two ways: static superficial (SSF) and submerged (SF).
From a historical point of view, static fermentation was the first acetification method. Thanks to applied technology to biochemical process, the evolution brought a new paradigm switching from static state to a dynamic state of the raw materials in order to increase the metabolic activity of the AAB mainly triggered by increased level of oxygen available.
As previously said, the AAB are strictly aerobic bacteria; technology applied to the fermentation has increased the availability of oxygen in the medium moving from a static regime, where fermentation was possible only on the surface of the liquid, to a dynamic regime where the fermentation wasn't just on surface but "submerged". For this reason, the more recent and evolved method is called "submerged fermentation" which is used to produce vinegar at industrial scale. The submerged method has been the first dramatic enhancement of vinegar fermentation process from an efficiency point of view (Ebner et al., 1996).

2.3.2 Static superficial method

SSF is one the oldest way for vinegar production, laying on a boundary area between not voluntary (spontaneous) process and first fermentation activity managed by humans as soon vessel and others containers has been available.
The so-called surface culture fermentation, where the AAB grow on the air–liquid interface in a direct contact with atmospheric air (oxygen). The presence of the bacteria is limited to the surface of the acidifying liquid and hence, it is also considered as a static method. Nowadays, this method is employed for the production of traditional and selected vinegars and a very long period of time is required to obtain a high acetic acid degree. As a consequence, production time and costs are higher respect those produced by submerged system.
Nowadays SSF is still practiced by families and small artisanal companies due to the low level of technology involved into the process.
As seen in chapter 1, this method has been chosen from Italian regulatory organism, as a discriminant system and feature between industrial and artisanal production.
The study in this thesis aims to investigate if the application of this fermentation method, could represent a possible competitive advantage for small companies in search of higher market position.

2.3.3 Submerged fermentation

SF for vinegar production is an aerobic process by which the ethanol in liquids such as spirits, wine or cider is oxidized to acetic acid by AAB, in controlled stirring conditions (Ebner et al., 1996).
In order to achieve faster rates of vinegar production, the first submerged methods adopted were meant to increase the acetification surface contact. These techniques are applied in the elaboration of vinegars by the Luxemburgian and Schutzenbach methods or by Quick vinegar processes methods. Basically, these methods employ inert materials as a bacterial supporting medium and the acetification rate is relatively high. As oxygen requirement is high, additional supply is provided by rotating the cask in the case of the Luxemburgian method whilst in the Schu¨tzenbach method the acetifying liquid is pumped through the fixed wood shaving bed which is supported by a false base. The acetifying wash is trickled down over the support against a counter current of air and in this way the acetifying stock recirculates until the desired acetic degree is achieved. These are intermediate processes between spontaneous and passive (empirical) fermentations and the latest technologies where forced aeration is involved.
The submerged culture system implies that the AAB are suspended in the acetifying liquid in which a strong aeration is applied to assure the oxygen demand. This method was introduced for the production of vinegar at the beginning of XX century.
This system basically consists of stainless steel fermentation tanks with a capacity of 10,000–40,000 l, air supply system, cooling system, foam controlling system, loading and unloading valves.

The basic requirements for submerged processes are the availability of appropriate alcoholic stocks, uninterrupted aeration and AAB strains tolerating high concentrations of acetic acid and ethanol, which are not sensitive to phage infections and that require small quantities of nutrients, to produce high amounts of acetic acid (Ebner et al., 1996). One of the most important features of the bioreactors is the aeration system. This system consists of a hollow body turbine supported by a non-rotating stator. The turbine sucks air from the outside and releases it into radial holes that open in the opposite direction of rotation; the action of turbines results in very fine air bubbles and homogenous air–liquid dispersion. The air–liquid emulsion is pushed upwards and diverted by

deflectors. All of the mass is maintained in a constant state of agitation to prevent the formation of low oxygen tension areas, which are unfavorable for the AAB activity (Ebner et al., 1996).

A submerged system has several advantages over other techniques (e.g. Solid state Fermentation and surface fermentation), including high yield and process speed. Over the last few decades, many studies have examined process variables (oxygen availability, temperature, acetic acid and ethanol content), and a number of strategies for process control have been established.

SF at the industrial scale is primarily performed in a semi-continuous mode (known also as repeated batch). Close to substrate depletion, a fraction of the fermentation broth is withdrawn and replenished with fresh medium. Thus, the residual culture broth of the preceding batch works as inoculum of the subsequent batch (Gullo et al., 2014). The main advantage of repeated batch processes is the increased productivity compared with usual batch processes because of shorter cycle times.

Repeated batch processes may be superior to continuous cultivation, for instance, in biological systems which suffer from a severe product inhibition such as acetic acid inhibition in vinegar production. The high ethanol and acetic acid concentrations in vinegar production act as auto-selective for AAB. Hence, the process can be conducted in an unsterile system.

According to previous studies, the indigenous bacterial population, studied by cultivation methods, during SF for vinegar production appears quite homogeneous, as it is mostly composed of the genus *Komagataeibacter* and, in some cases, *Acetobacter*.

2.4 Acetic acid bacteria and bacterial cellulose production

Bacterial cellulose (BC) is the main active component of the biofilm produced by AAB, which are well known as biofilm producer organisms. Considering the theme of this PhD thesis, BC is both an undesired product of AAB metabolism and a value added for some emerging vinegars/beverages.

For the majority of vinegars the occurrence of BC is the result of irregular fermentations, providing technological drawbacks and anomalous sensorial characteristics of the vinegar as final product (Gullo and Giudici 2008). However, the emerging consumer's demand for artisanal foods and beverages, makes BC a positive attribute in some products.

Moreover, raw materials such as fruit juices, vinegar and kombucha tea are suitable sources for detecting AAB strains able to produce BC. The recovery and characterization of AAB strains able to produce BC is a current need of the biotechnology industry. From all these observations, below an outlook on BC synthesis and applications, is provided.

BC is an adaptable biopolymer that can be used in a large variety of applications, including food (e.g. noncaloric bulker), pharmaceutical and biomedical products (e.g. cosmetics, skin substitute for burn wounds) and engineering (e.g. diaphragms for electro-acoustic transducers, paint additives, coatings and as reinforcement material) (Jonas and Farah 1998; Gullo et al. 2018). The global BC market is estimated at 207.36 million USD in 2016 and is estimated to reach 497.76 million USD by the 2022 (QYResearch Reports 2017).

By the way, BC production at industrial scale is limited by the low yield and high production cost. The availability of appropriate AAB strains and the optimization of both culture media and the production process seem to be main elements to overcome these limits (Gullo et al. 2017; Ruka et al. 2012; Kuo et al. 2015).

To reduce the cost of BC production, different strategies were tested using low costs and waste products as carbon sources. High BC productivity was obtained using molasses from corn steep liquor (CSL) added with citric acid in *K. xylinus* BCR5 culture. Second hand fruits, which are not marketable, are also considered potential sustainable raw materials for BC production.

The most investigated methods to produce BC comprise static and submerged regimes by which uniform smooth gels and spheres can be obtained, respectively (Islam et al. 2017).

The high suitability of BC use in different fields can be attributed to its physico-chemical and mechanical properties. BC has higher surface area than plant cellulose and is a very malleable material. Native BC does not require purification steps that can cause alterations of its properties.

The ability to acquire flavors and colors makes BC a suitable adjuvant for foods and beverages. As a food additive, it is used for its gelling and thickening properties. BC is used in the manufacturing of nata de coco and Kombucha tea. Nata de coco is a Philippine dessert produced from fermented coconut water. The obtained BC is cut into tiny sections and immersed in syrup of sugar (Iguchi et al. 2000), whereas Kombucha tea, is a beverage obtained from sugared tea fermented by yeasts and AAB (Mamlouk and Gullo 2013). In addition, BC is used in low cholesterol diet, thanks to serum lipids

and cholesterol-lowering effect (Chau et al. 2008). Moreover, BC with its hydrogel-like structure could be a new material for salads and low-calorie desserts. (Keshk and Sameshima 2015).

In medicine, BC is used as artificial skin, artificial blood vessels, and hemostatic materials. One of the most important uses of BC in biomedical field is as wound healing scaffolds (Picheth et al. 2017). During the last years, many brands (such as Biofill®, Gengifill®, Bionext®, and Xcell®) produced BC devices that can be used in a wide variety regenerative medicine application. Different effects, such as pain relief, fast skin regeneration and reduction of inflammatory response characterize these devices (Rajwadee et al. 2015). Thanks to the stabilizing effect of oil-water emulsion, low toxicity, and ability to hydrate the skin without the need of surfactants, BC is extensively used in cosmetic field for facial mask creams and as a powder in facial scrubs in association with other natural materials (as olive oil, Vitamin C, Aloe vera extract, and powdered glutinous rice). An emerging BC application in cosmetic is the production of contact lenses, due to its transparency, light transmittance, and permeability to liquid and gases (Ullah et al. 2016).
Among emerging products from AAB, for sure bacterial cellulose have received increasing attention because of its high potential in a wide array of applications, but actually the low production yield and high cost, limit the development of large-scale processes. However, the wide applied research in this area is providing the basic platform for optimization of industrial biopolymers processes using AAB.

2.5 Acetaia San Giacomo company's productive set up

2.5.1 Company profile

The company was established in 1999 starting from existent set of barrels (110) for PDO Traditional Balsamic Vinegar of Reggio Emilia (TBVRE), taken over from the familiar production (Figure 2.1 and 2.2).
Since the beginning the productive apparatus has been designed with an artisanal approach, focusing only on static surface acetic fermentation and very long aging processes.
Nowadays the company has 740 barrels certified for PDO TBVRE and 100 more for artisanal vinegars (Fig. 2.3) made accordingly to the new subcategory requirements.

In 2010 the company has been awarded with a gold medal from the local Chamber of Commerce, for "Excellent Company" having reached to different achievement like: market positioning, innovative project, average age of the employees.
Is a "Teaching location" (Sede Didattica) for University of Gastronomic Science of Pollenzo.
The company's aging rooms are visited yearly by hundreds of people from all over the world, interested in high quality food.

Figure 2.1. Set of Barrels for PDO TBVRE

Figure 2.2. Set of Barrels for PDO TBVRE

Figure 2.3. Barrels for static superficial fermentation of vinegars

Together with other small artisanal producers, starting from 2014 the company contributed with the Parliamentary legislator to create the new regulatory space for artisanal vinegars protection.
Raw materials are all certified organic and from 40 to 50% of them are provided by directly farming of local vineyards.
Market positioning of the company is in niche-luxury segment due to the average sell-out premium price of the products.
Averagely, more than 70% of production has always been exported.
Despite the traditional production, the company always aimed to differentiate the production in order to adapt its offer in a highly competitive scenario.
The first shift in market sector was performed in the last 10 years of activity, moving from gourmet food retailers to food service.
The food service sector is growing globally while the retail food market is shrinking and is unable to convey the high value added, connected with artisanal products.
The higher cost implicit in premium products for food preparation, on the other hand, has a lower impact in food cost composition. Moreover, the leverage effect of premium food vinegars and condiment can be highly appreciated from the operators that willing to differentiate their offer.

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Date: 01 Oct 2019
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Chapter 3

DESIGNING OF KIT FOR VINEGAR PRODUCTION AT DOMESTIC SCALE BY NOT PROFESSIONAL USERS

CHAPTER 3. DESIGNING OF KIT FOR VINEGAR PRODUCTION AT DOMESTIC SCALE BY NOT PROFESSIONAL USERS

3.1 Introduction

3.1.1 The growing trends related to self-production

During its business activity and public's relations, San Giacomo company was able to detect an increasing need to produce vinegars or acetic-based products on a domestic scale by not professional operators. Customers frequently has been asking for starter cultures, how-to advice and tips to make vinegars by their own.

Production of vinegars, with leftover wine or other fermented liquid, has been a domestic economy activity very common in the past among many countries.

This trend reflects the growing demand for greater sustainability through a commitment to recycling, reuse and no-waste approach.

Not only private consumers are interested in self production of vinegars but also restaurant owner and chefs. Over the past 20 years, restaurant kitchens have been equipped with industrial-derived equipment that has become increasingly affordable and applicable to restaurant-scale production.

Consumer participation in value creation activities has been assessed with different terms and facets, such as prosumption (Ritzer and Jurgenson 2010; Xie et al., 2008), coproduction (Etgar 2008; Le MeunierFitzHugh et al., 2011), self-service (Collier and Kimes, 2013; Collier and Sherrell, 2010), do-it-yourself (Williams, 2008; Wolf and McQuitty, 2011), customer engagement (Vivek et al., 2012), and cocreation of value (Vargo and Lusch 2004).

Different control factors, such as time, skills and knowledge, money, and cooperation by others, are suggested among important factors for consumers to participate in producing a product or service.

In general, self-production activities can be motivated by intrinsic values (e.g., fun, playing), extrinsic values (e.g., excellence, self-expression), or social benefits (e.g., social status, social esteem) (Etgar, 2008).

The research then identifies two sectors related to vinegars consumption where the production can be carried by not professional user:

- Private consumer

- Restaurateurs (and small agri-food business owner)

3.1.2 State of the art of technology for small scale acetic fermentation

A market research has been carried out to define the state of the art of the equipment for small-scale acetic fermentation process.

The smallest and more advanced machine (able to carry out autonomously a complete acetic fermentation) is a laboratory-scale fermenter, adopting submerged culture fermentation process.

The process control of the pilot fermenter is designed for the standard-batch (semi-batch) fermentation for the production of alcohol vinegar with up to 14% acidity as well as for the production of wine and cider vinegar. A user-friendly touch panel enables an easy operation. Fermentation parameters such the alcohol and acetic acid concentrations are measured online, often with devices under patent status. As the oxidation of ethanol into acetic acid is an exothermic process, cooling is one part of the equipment.

The fermenter vessel has a minimum capacity of 6 lt (pilot fermenter) and a potential yearly production of 1.000 lt (based of vinegar of 10% of acetic acid content). The price for the pilot fermenter is approximately 20.000 Euro and the price for the automated control system (based on ethanol concentration measurement) is approximately 10.000 Euro.

The cheapest equipment for a domestic scale acetification process is a glass demijohn with larger open mouth on top. Ceramic pot and vase are also available. Size range goes to 5 l to 25 lt. Price is less than 100 Euro.

To enable private consumer in vinegar self-production, starter culture (mother of vinegar) in liquid state are also available on the market. The efficiency of these starter cultures is not guaranteed.

Although many semiautomatic equipment and tools have been introduced for the self-production of private consumers (for example bread machine, homemade beer kit, microgreen kit), we have observed that there are not available devices and or kits for the needs of vinegar non-professional users. Affordable equipment capable of conducting the fermentation process at domestic scale by increasing the chances of success, are not currently available on the market.

Then, this part of the research aims at acquiring the technological progress of the vinegar production system. In particular, the objective is the development of a vinegar production system that can be adopted by non-specialized users.

In this context, the process of "domestic acetification" means a process of acetic fermentation carried out in domestic or micro-business (restaurant) environment, for small quantity output (micro-batch of 3-5 liters).
The concept of “Kit” in this section is meant as an array of knowledge and tools that will help in start, control and finalize the process of acetic fermentation.
A peculiar feature of these processes is the execution of procedures by non-specialized users and the use of highly variable raw materials in their composition.
The user potentially interested in developing a process of domestic fermentation should have basic technical and scientific knowledge. Aim of the research is to provide them the basic know how and basic tools to successfully carry the fermentation process.
The raw materials being studied are liquids already subjected to alcoholic fermentation such as wines. From a microbiological point of view, vinegar production was evaluated by mixed acetic cultures (AAB present in liquids and in fermentation environments) and by a selected starter culture.
Three Critical Points (CP) for domestic acetification has been identified:

- Start up procedure;
- Control system (process and finalized output);
- Speeding up system.

As far as production technology is concerned, the processes were mainly designed on surface static system fermentation method. Some tests replicating the submerged system have been also carried out in order to speed up the process.
The outline of this research was designed in the frame of the project “*Produzione di un sistema di acetificazione innovativo - Acquisizione di servizi di ricerca e sperimentazione*” (Contributo Regione Emilia Romagna. Decreto del Commissario n. 393 del 13/03/2014) of Acetaia San Giacomo Company in collaboration with the Department of Life Sciences of the University of Modena and Reggio Emilia.

3.2 Materials and methods

In Figure 1 a schematic outline of the detected needs and the experimental steps are depicted.

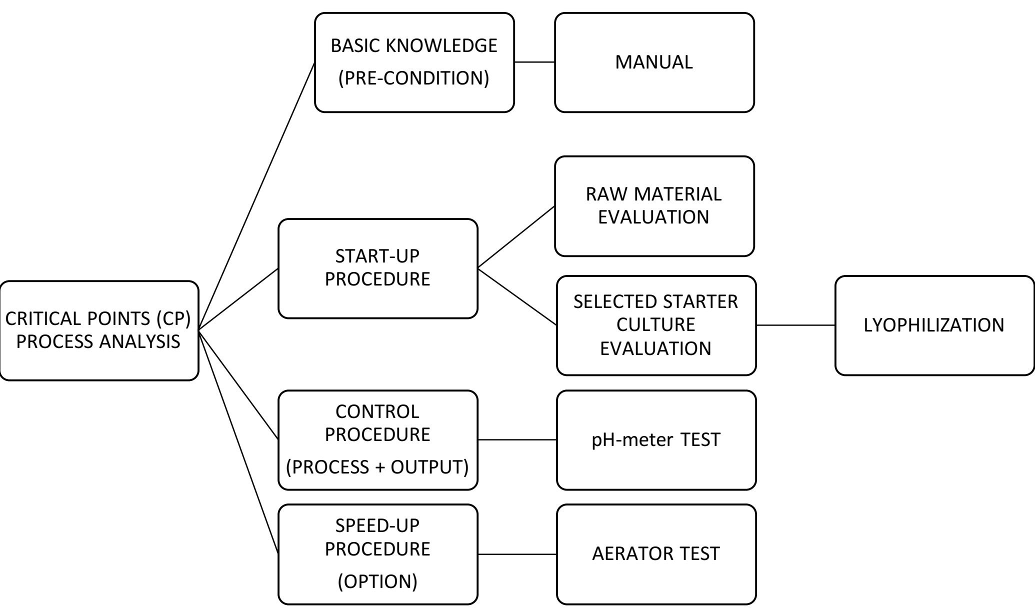

Fig. 1. Schematic outline.

3.2.1 Raw materials and end products recognition

To assess suitable fermentation indicators a set of raw materials and end products were selected. Raw materials (wines) that can be found as waste, at domestic scale, such as wine residues, bulk wines and wines in which the acetic fermentation were chosen. The raw materials were grouped as follow:

1) Wines (different types of wine used for the acetic fermentation);
2) fermenting wines (wines in which the acetic acid fermentation was started in superficial static system). Instead, end products were vinegars available on the market that were produced with different fermentation systems.

3.2.2 Bacterial strain, cultivation, storage

In this study the acetic acid bacterium strain UMCC 1754 (*Acetobacter pasteurianus* species) of the Unimore microbial culture collection (UMCC, www.umcc.unimore.it) was used. The strain, stored

at -80 °C, was rehydrated in 5 mL of GY broth (glucose 5% w/v; yeast estract 10% w/v) and cultivated aerobically at 28 °C for 5 days.

3.2.2.1 Selected starter culture scaling-up in static system

The starter culture from the strain UMCC 1754 was implemented by superficial static system acetification (Figure 2). Briefly, 1 mL aliquote from rehydrated strain culture was used as inoculum in 5 mL of GY broth. After five days cultivation, the culture was transferred in 100 mL flask, using 50 mL of GY broth. The starter culture was obtained by a scaling-up procedure, using 2 L flasks with a working volume of 50 mL. Periodially, not filtered wine was added to the culture, in order to maintain always the value of ethanol and tritatable acidity (7-8% p/v) in the range (2.5-3.5% v/v) respectively.

Fig. 2. Starter culture (UMCC 1754) scaling-up in static system.

3.2.3 Innovative process design and development for vinegar production with and without the selected starter culture

For the acetic fermentation in superficial static system, two trials were performed. We will refer to the two trials as "Vin_ind" (indigenous fermentation) and "Vin_Start" (selected starter fermentation). The two trials were developed using 5 L tanks, with the following working volume:

- "Vin_Ind"- 2.5 L of commercial wine
- "Vin_Start"- 2.05 L of commercial wine + 0.45 L of selected starter culture

The processes were conducted at room temperature. In order to monitor the analytic parameters, all the measures were performed in triplicate using a time intervals of 3 days. The analytic parameters included titratable acidity and pH.

3.2.4 Aerator description

To evaluate the effect of the aeration on the acetic fermentation process, the aerator "C Top King" was used. The device is made up a small pump (Figure 3 A) taking the air from the environment. Through a tubes series, the airflow was supplied to the fermenting substrate. At the end of the suppling tube, a stainless steel diffusor was added (Figure 3 B), allowing the micro-bubbles formation.

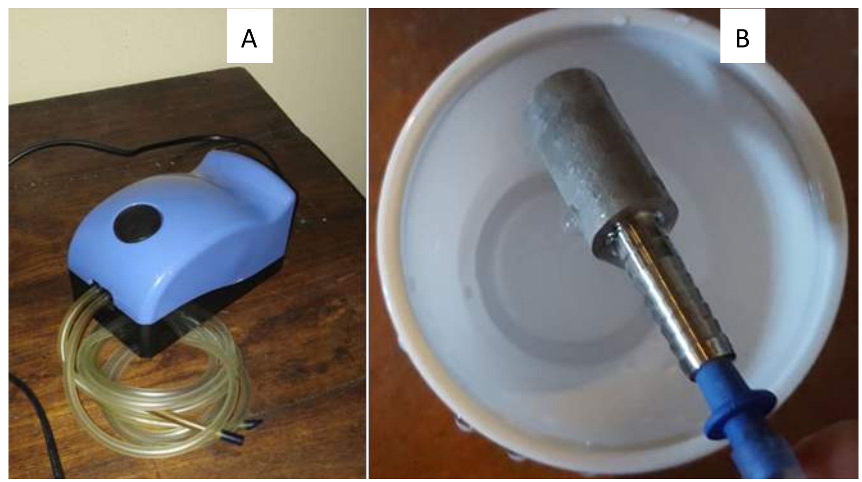

Fig. 3. "C Top King" aerator A) Pump B) Stainless steel diffusor.

3.2.5 Acetic acid fermentation tests by forced aeration

The acetic fermentation was carried out using a start mixture (5 L) obtained from commercial wine and the starter culture from *A. pasteurianus* UMCC 1754, in a ratio of 3:1. The analytical parameters like pH, ethanol, dissolved oxygen and titratable acidity (expressed as acetic acid) were measured. The fermentation test was performed using 3 flasks and 3 beakers whit a total volume of 1 L in which 500 mL of the mixture was added. The trials were named as: "Trial A", "Trial B" and "Trial C" as described in Table 1. The analytical parameters were measured every 3 days. In the "Trial A", the aerator was used at the beginning of the trial (T0) for all the testing period; in the

"Trial B", the aerator was used starting from day 3; in the "Trial C" the aerator was not used, performing the trial in static conditions.

Vessel	Trial A	Trial B	Trial C
Flask 1 L	Aerator T0	Aerator T3	Static system
Beaker 1 L	Aerator T0	Aerator T3	Static system

Table 1. Experimental design of acetic fermentation tests.

3.2.6 Analytical parameters determination

3.2.6.1 pH, titratable acidity and ethanol

pH and titratable acidity were measured using an automatic titrator (TitroLine®EASY) equipped with an SI Analytics electrode (Figure 4 A). Samples were neutralized with NaOH (0.1 N) at pH 7.2. It was assumed that all sample acidity was due to acetic acid. The acidity value was expressed as grams of acetic acid/100 mL. Ethanol was measured by ebulliometric analysis using the Malligand device (Figure 4 B).

Fig. 4. Basic devices for fermentation monitoring: A. Automatic titrator TitroLine easy; B. Malligand ebulliometer.

3.2.6.2 Dissolved oxygen determination

The percentage of dissolved oxygen was measured by using an ossimeter supplemented with a microprocessor HI 2400 (Hanna Instruments). The device was made up by:

• D.O. probe, HI 76407/2

• 2 replacement membranes, HI 76407A

• Electrolyte solution HI 7041S, 30 mL

• Power supply 12Vdc, HI 710006

The oxygen probe was equipped with a PTFE semipermeable membrane that cover a polarography sensor and a thermistor for temperature compensation.

3.2.6.3 Optical microscopy observations

Cell morphology and direct observation of samples were carried out by optical microscopy using C. Zeiss microscope apparatus (Axiolab) at magnitude 40X and 100X with the oil immersion lenses.

3.3 Results and discussion

3.3.1 Raw materials suitable for the development of fermentation processes and analysis of end products from the market

Different raw materials and end products were selected and analyzed to the aim to provide appropriate indicators of the fermentation trend.

The alcoholic liquid to produce vinegar should possess all the features required for promoting the acetic acid fermentation. In this study, in order to set-up the acetic fermentation process in non-industrial conditions, wine as the waste substrate that can be found in every domestic environment was considered. However, waste wines can have high variability in terms of ethanol content, traces of acetic acid due to an uncontrolled storage once bottles are opened, they can contain residual yeast cells and a variable amount of stabilizing compounds such as sulfur dioxide. A higher amount of stabilizers can affect the acetic fermentation, inhibiting AAB growth, in particular if indigenous AAB cultures are used (Gullo et al., 2014).

The pH values, ethanol and titratable acidity (expressed as acetic acid content) of raw materials are reported in Table 2. These parameters were chosen as indicators of the starting and/or the end acetic acid fermentation. The pH of the samples ranged from 4.03 (wine) to 2.73 (vinegar); titratable acidity ranged from 0.32% (wine) to 8.13% (vinegar); the ethanol content ranged from 14.8% (wine) to 1.90% (vinegar).

Simultaneously, the vinegars from the market as end products that came from high variable raw materials and different production techniques, were assayed for pH, titratable acidity and ethanol content (Table 3).

All of the assays performed suggest the suitability of pH as indicator of transformation of wine into vinegar. The data obtained on wine and vinegars, highlight the correlation of pH values and titratable acidity. However, other than acetic acid, in the sample of this study, also different organic acids than acetic acid, like gluconic acid, tartaric acid, malic acid, citric acid and succinic acid can occur. For this reason, variations of pH cannot be considered uniquely as indicator of the acetic acid content, but they can provide information of the fermentation trend. From these considerations, we can assume that pH can release user-friendly and preliminary information of the fermentation status to the not specialized user.

Product	**pH**	**Titratable acidity (g/100 mL)**	**Ethanol (% v/v)**
Wine			
White wine	3.40±0.02	0.41±0.01	10.0±0.7
Red wine	3.33±0.01	0.71±0.04	14.8±0.6
Malvasia wine	3.33±0.06	0.62±0.03	12.7±0.8
Dry wine	3.36±0.08	0.62±0.02	9.40±0.4
Wine (Lambrusco) Batch 1	3.50±0.04	0.36±0.06	8.50±0.5
Wine (Lambrusco) Batch 2	4.03±0.03	0.36±0.07	9.70±0.7
Wine (Lambrusco) Batch 3	3.96±0.07	0.32±0.05	8.50±0.6
Wine (Lambrusco) Batch 4	4.00±0.06	0.37±0.02	9.60±0.8
Fermenting wine*			
Fermenting wine T0	2.90±0.05	6.99±0.05	3.28±0.5
Fermenting wine T1	2.89±0.09	7.38±0.03	2.50±0.7

Fermenting wine T2	2.86±0.03	7.80±0.01	2.43±0.6
Fermenting wine T3	2.78±0.02	7.84±0.04	0.60±0.9
Fermenting wine T4	2.77±0.08	7.69±0.02	2.70±0.4
Fermenting wine T5	2.73±0.03	8.13±0.02	1.90±0.7
Fermenting wine T6	3.04±0.04	4.87±0.03	3.10±0.6

*superficial static system

Table 2. Analytical parameters of raw materials (wine and acetifying wine).

N.	Product	Titratable acidity on the label (g/100 mL)	Analytical parameters		
			Titratable acidity (g/ 100 mL)	pH	Ethanol (% v/v)
1	Red wine vinegar	/	8.66 ± 0.07	3.33 ± 0.07	1.90±0.5
2	Lambrusco wine vinegar		9.32 ± 0.01	3.26 ± 0.02	1.60±0.6
3	Beer vinegar	/	4.41 ± 0.04	3.42 ± 0.06	0.70±0.8
4	Quince vinegar	/	5.60 ± 0.03	3.62 ± 0.04	0.40±0.9
5	Vinaigre de Banyuls	6.0	6.79 ± 0.04	3.16 ± 0.09	0.80±0.7
6	Timorasso wine vinegar	/	8.03 ± 0.15	2.95 ± 0.04	0.50±0.6
7	Wine vinegar	10.5	11.52 ± 0.14	3.18 ± 0.04	0.00
8	BalsaMela (San Giacomo company)	/	3.43 ± 0.09	3.64 ± 0.02	0.00
9	Honey vinegar	/	6.20 ± 0.09	2.78 ± 0.04	0.30±0.8
10	Wine vinegar	/	2.10 ± 0.13	3.83 ± 0.03	0.30±0.9
11	Amarone wine vinegar	6.0	6.51 ± 0.02	3.31 ± 0.03	0.00

12	Malvasia vinegar	6.0	6.26 ± 0.02	3.24 ± 0.03	0.90±0.5
13	White wine vinegar	6.0	6.28 ± 0.05	3.26 ± 0.02	0.10±0.3
14	Apple vinegar	5.0	5.45 ± 0.04	3.22 ± 0.04	0.20±0.2
15	Balsamic vinegar of Modena	6.0	6.14 ± 0.59	3.34 ± 0.03	0.35±0.3

Table 3. Analytical parameters of vinegar samples available on the market.

3.3.2 Design and development of acetic fermentation assays using selected starter cultures and indigenous cultures

The applications of AAB starter cultures are well documented in literature were they are described to be very useful in both static and submerged system (Sokollek e Hammes, 1997; Gullo et al., 2009; Zanichelli, 2014; Gullo et al., 2016). Although the handling and maintenance of the starter culture is expensive, their use provide numerous advantages in the fermentation process. For example, it is possible to select strains based on the ability to be thermo-tolerant or resistant to high concentration of acetic acid (Saeki et al., 1997; Trček et al., 2007). One of the main factors that need to be mentioned about the starter culture maintenance is the preservation of the biotechnological traits of strains, in order to guarantee the stability of the process and the product over time (Gullo et al., 2012; De Vero et al., 2006). The accumulation of mutations during the life cycle of bacteria may cause the lost of phenotypic traits like ethanol oxidation, acetic acid resistant, cellulose production and so on (Azuma et al., 2009, Gullo et al., 2012). The phenotypic instability is considered one of the main limits of starter culture development in vinegar production. For these reasons fermentation processes are generally conducted with mixed acetic acid cultures.

In this study based on the considerations above, the static system was chosen for the vinegar production. Furthermore, considering the ability of AAB in acetic acid production and considering that the speed of production is directly correlated to the oxygen concentration, two different set-up of the aeration system were chosen. In order to implement all the component of the Kit, the acetic fermentation process was assessed using both indigenous and selected AAB cultures.

The assay using the selected starter culture was conducted with the microbial strain *A. pasteurianus* UMCC 1754 that was chosen for its genetic stability, robustness, high oxidative performance in both static and submerged systems and inability to produce bacterial cellulose from different carbon sources. The genetic and phenotypic stability were previously evaluated after 10 years of cryoconservation at -80 °C in the UMCC collection (Gullo et al., 2012). Furthermore, *A. pasteurianus* UMCC 1754 was used in a combined static and submerged system with high sugar concentrations (Zanichelli 2014; Gullo et al., 2016). Phenotypic and technological traits of the strain UMCC 1754 are reported in Table 4. Moreover, UMCC 1754 showed high genetic and phenotypic stability when the fermentation was conducted under uncontrolled environment, using non-sterile raw materials (Zanichelli 2014).

Trait	**Value**
Form	Rod
Gram +/-	-
KOH reaction	+
Catalase reaction	+
Water soluble pigments	-
Acetic acid production	+
Acetic acid oxidation to CO_2 and H_2O	+
Growth feature	
Ethanol	-
Methanol	-
Medium growth:	
GYC	+
AE	-
YPM	+
RAE	-
Frateur	+
Passmore and Carr modified	+
Carr	+
Cellulose production	-

D-glucose-based medium growth (%)	
20	+
25	+
30	-
Fermentation system:	
Superficial static system	+
Submerged system	+

Table 4. Phenotypic and technological traits of *A. pasteurianus* UMCC 1754.

[a]DSMZ 11825^T (*A. pomorum*) was used as control

[b]DSMZ 2004 (*K. xylinus*) was used as control. Data from Gullo et al., 2012; Gullo et al., 2016.

To test the spontaneous fermentation of wine into vinegar by environmental AAB, assays were performed using wine not inoculated with the starter culture. In Table 5 the main parameters of wine is reported.

The analytic parameters for the two theses ("Vin_Ind" and "Vin_start"), were measured every 3 days and reported in Table 6.

Substrate	**pH**	**Titratable acidity (g/100 mL)**	**Ethanol (% v/v)**
Commercial wine	3.50	0.50± 0.03	10.50

Table 5. Analytic parameters of the commercial wine.

Considering Vin_Start fermentation trend, an adaptation phase of the microbial culture can be observed from the slow acetic acid increase during the first 6 days. This phenomenon has been previously observed both at laboratory and industrial scale when AAB selected starter culture are used (Gullo et al 2009). The main increase of acidity was observed at time-point 9 days (T3) indicating the start of fermentation, consistent with a reduction of ethanol. The ethanol determination was performed consequently to the acetic acid increasing, verifying only the effectively conversion of ethanol into acetic acid. Microscopic observations highlight the presence of rod-shape bacteria. The presence of yeasts cells was not detected. The appearance of a superficial thin layer (T4; 12 days) corroborated the active fermentation in Vin_Start trials (Figure 5).

On the basis of these results the stability of UMCC 1754 for vinegar bioprocesses was confirmed. In the tests of wine without AAB inoculum (Vin_Ind), the decrease of ethanol was observed, but it was not correlated to the increase of acetic acid. The reduction of ethanol was mainly due to the evaporation process. Macroscopic and microscopic observations highlighted no superficial biofilm and the presence of yeasts, confirming that the acetic fermentation process was not occurred.

	Vin_Start			**Vin_Ind**		
Time	**Titratable acidity (g/100 mL)**	**pH**	**Ethanol (% v/v)**	**Titratable acidity (g/100 mL)**	**pH**	**Ethanol (% v/v)**
T0	4.30 ± 0.03	3.22		0.50 ± 0.03	3.50	10.50
T1	4.54 ± 0.05	3.22		0.50 ± 0.02	3.44	-
T2	5.13 ± 0.09	3.05		0.50 ± 0.01	3.42	-
T3	6.51 ± 0.04	2.93		0.50 ± 0.02	3.41	-
T4	7.28 ± 0.06	3.04	1.40	0.50 ± 0.015	3.39	-
T5	7.39 ± 0.08	3.07	-	0.50 ± 0.01	3.39	8.70
T6	7.30 ± 0.09	3.03	-	0.50 ± 0,03	3.39	--

Table 6. Analytic parameters of Vin_Start and Vin_Ind over time.

Fig. 5. Superficial thin-layer in Vin_Start sample after 12 days of cultivation.

Contrarily to static fermentation, mixed cultures operating in submerged system are successful used at industrial scale. In these systems, the high selective pressure due mainly to acetic acid, allows only the growth of a low number of different AAB, able to tolerate physiological stressors.

Instead in uncontrolled systems, were the raw materials are largely heterogeneous and the selective pressure is low, the beginning of the oxidative process is uncertain and very slow. Then, selected AAB cultures are necessary in the light to perform homemade fermentations by not specialized users.

3.3.3. The aerator effect on the fermentation process

In order to test the effects of the aeration system on the fermentation speed, three different assays, using the selected starter culture previously set up and wine, were implemented (Table 7). The experiments were conducted in two different vessels (beakers and flasks) to the aim to evaluate the best shape suitable for the kit (Figure 6). For each assay, three different conditions were tested:

A. Whit aerator (T0)
B. Whit aerator (T2, after 3 days)
C. Static system (without aerator)

   During the assays (A, B, C), the analytic monitoring of titratable acidity, ethanol reduction caused by oxidative fermentation into acetic acid, was performed (Tables 8 and 9).

For tests conducted in beakers suitable values of titratable acidity were obtained for all the conditions tested (A, B and C), since the values were consistent with the required standards of vinegar as final product. However, interesting differences according to the forced aeration supplied were detected. The highest tritatable acidity (8.94%) after 9 days cultivation (T4) was observed in trial B, where forced aeration was started at T2 (after 3 days). Whereas the lowest acidity (7.59%) was reached in the trial C for which the fermentation was conducted without supply of air. Intermediary results were observed for trial B (acidity 8.13%). All the pH trends were consistent with the increase of acidity during fermentation (Table 8).

Considering ethanol, the depletion was total in trials A and B, whereas a residual content (2.4%) was observed in trial C conducted without forced aeration. Although the effectiveness of the fermentation was achieved, it is clear that the forced aeration promoted a loss of ethanol by evaporation.

Fig. 6. Fermentation tests with forced aeration in flask and beakers.

Substrate	**Volume (L)**	**Titratable acidity (g/100 mL)**	**Ethanol (% v/v)**	**pH**	**Dissolved oxygen (%)**
Starting solution	3.0	6.00± 0.03	4.50	3.29	18.2

Table 7. Analytic parameters of starting solution.

3.3.4 Acetic fermentation in beaker

BEAKER (500 mL working volume)					
Time	**Titratable acidity (g/100 mL)**	**Ethanol (% v/v)**	**Oxygen (% dissolved)**	**pH**	**Operation**
Sample "**A**"					
T0	6.00	4.5	18.2	3.29	
T1	7.59	2.6	50.0	3.27	
T2	7.65	1.4	~ 0.0	3.24	
T3	7.81	1.0	32.0	3.20	+ 50 mL wine

T4	8.13	0.4	~ 0.0	3.18	
Sample "**B**"					
T0	6.00	4.5	18.2		
T1	7.59	3.0	~ 0.0	3.24	Starting aeration
T2	7.76	2.0	55.0	3.22	
T3	7.91	1.8	20.0	3.18	+ 50 mL wine
T4	8.94	0.3	~ 0.0	3.14	
Sample "**C**"					
T0	6.00	4.5	18.2		
T1	6.71	3.4	~ 0.0	3.27	
T2	7.53	2.5	5.0	3.24	
T3	7.58	2.4	3.0	3.21	+ 50 mL wine
T4	7.59	2.4	~ 0.0	3.23	Superficial thin-layer

Table 8. Acetic fermentation assays in beaker.

3.3.5 Acetic fermentation in flasks

Flask assay (500 mL working volume)					
Time	**Titratable acidity (g/100 mL)**	**Ethanol (% v/v)**	**Oxygen (% dissolved)**	**pH**	**Operations**
Test "**A**"					
T0	6.00	4.5	18.2		
T1	6.99	3.3	45.0	3.24	
T2	7.17	n.d	32.0	3.21	
T3	7.77	2.0	3.0	3.18	+ 50 mL wine

T4	7.62	1.3	0.0	3.25	
Test "**B**"					
T0	6.00	4.50	18.2		
T1	6.83	4.30	~ 0.0	3.29	Starting aerator
T2	6.93	3.70	55.0	3.25	
T3	6.98	3.70	32.0	3.26	+ 50 mL wine
T4	7.06	3.50	~ 0.0	3.24	
Test "**C**"					
T0	6.00	4.50	18.20		
T1	6.22	4.30	~ 0.0	3.33	
T2	6.48	4.00	10.0	3.24	
T3	6.18	4.30	7.00	3.26	+ 50 mL wine
T4	8.01	2.40	~ 0.0	3.02	Superficial thin-layer

Table 9. Acetic fermentation in flasks.

Also the tests conducted in flasks allowed to reach titratable acidity values satisfying the legal value for vinegar (≥6%) and pH trends in accordance with the acidity increase (Table 9). However, for all the trials the values of acidity was lower respect those obtained in beakers. This can be due to the different surface/volume ratio of vessels that is 13.85 cm^2 for flasks and 86.57 cm^2 for beakers. Despite the surface/volume ratio is less using flask than beakers, the decrease of ethanol during the first 9 days of cultivation was 0.3 and 0.4, which is comparable with the values obtained using beakers.

In the sample B, an increase of ethanol leaks was observed consequently the starting of aeration. Indeed, during the first 3 days of cultivation, when the aerator was switched off, the reduction of ethanol was only 0.2 %, while in the next days (T3), when the aerator was switched on, a leak of ethanol of 0.6 % was detected.

In trial C, which was conducted by static fermentation without supplied air, the ethanol consumption was less respect to tests A and B. Furthermore, the ethanol reduction was proportionally to the acetic acid increasing. After 9 days of cultivation (T3) the fermentation process was not started, while in the next 9 days the ethanol consumption increase rapidly, indicating that the AAB activity was high. Moreover, a superficial thin-layer due to the growth of the AAB culture was observed. This allowed to the further increase of acidity.

3.3.6 Dissolved oxygen (% DO) for both beaker and flasks assay

The trends of dissolved oxygen after 3 days of starting aeration were very high (more than 50%) in both sample A (T1) and sample B (T2). This behavior can be explained considering the correction functioning of aerator and the low metabolic activity of AAB, which were not completely adapted to the new environment conditions. After the adaptation, in both beaker and flasks assays were detected a dramatic reduction of dissolved oxygen, whit values near to the zero. This behavior can be explained considering the metabolic activity of the AAB culture, which oxidizes rapidly ethanol to acetic acid. The trends of analytical parameters confirmed that assumption, since low values of dissolved oxygen mean an increase of titratable acidity and consequently reduction of pH and ethanol. If we consider sample C in static condition, the dissolved oxygen was reduced referring to the begin value of 18.2 %, until values near to zero in the fermentation phase. The container shape seems does not affect the dissolved oxygen trend.

3.4 Conclusions

The results obtained from this study highlight the possibility that the fermentative process can be performed in superficial static conditions at laboratory scale (simulating domestic conditions), using different fermentative raw materials. By using only pH as parameters, that is not thorough, the users can obtain information on the different phase of the fermentation process. However, in order to understand when the final product can be considered ready, the titratable acidity should be taken into account. Moreover, higher variability among vinegars was observed considering the starting raw materials and the different fermentation systems.

From this study, the use of the selected starter culture has been show guarantee a rapid begin of the fermentative process. The microbial strain chosen in this study (*A. pasteurianus* UMCC 1754) was previously studied for its genetically and phenotypically stability. Also in this research the robustness of the strain was confirmed suggesting its use for large scale application. Furthermore, the strain was able to resist against stress factors like thermal variations, preserving its ability in the oxidative fermentation. Moreover, preliminary tests show that lyophilized starter can be considered, providing a more user-friendly procedure to make vinegar by not specialized users.

The acetic fermentation assays (sample A, B and C) were performed to understand if the use of the aerator can improve the oxidative speed. The results show that in samples with forced supply of air the fermentation process started easier than without. Despite the strain used was able to adapt itself to the environmental stressors, the high titratable acidity where detected when the aerator was working, after 3 days of the cultivation begin, highlighting the need of time for the strain adaptation. The values of the dissolved oxygen confirm this hypothesis. Since the fermentation using the selected starter provided suitable values of acidity both in aerated and not aerated tests, the use of aerator is a choice of the user, according the system which is more satisfactory for its need.

All the results collected in this study suggest the possibility to realize a kit for vinegar production at non-industrial scale system and suitable for non-professional users. This is possible integrating the kit with a starter culture of selected strain and a very simply user-manual.

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Chapter 4

DESIGN OF AN INNOVATIVE PRODUCT BASED ON WHEY, MADE WITH SCALING FERMENTATIONS

CHAPTER 4. DESIGN OF AN INNOVATIVE PRODUCT BASED ON WHEY, MADE WITH SCALING FERMENTATIONS

4.1 Introduction

Food industry is constantly growing and evolving thanks to innovative technologies that enable to satisfy consumer demand of high-added value products. Even the non-alcoholic beverages market has expanded considerably due to the real change in the idea of “drink”. Nowadays, consumers require a beverage to be not only refreshing but also healthy and ecologically sustainable. For centuries, in the East, kombucha tea has been consumed as a functional beverage. This fermented non-alcoholic drink is produced through tea leaves infusion and, subsequently, alcoholic and acetic fermentation (Sievers et al., 1995). The result is a sweet and slightly acidic tea. This beverage firstly appeared in Europe in the early 19th century and its consumption was commonly associated to several health benefits due to its composition (Table 1). Nowadays, kombucha tea has been confirmed by scientific studies to have such properties. Some reported effects of kombucha from tea drinkers’ testimony and Russian researchers are: detoxification of blood, reduction of cholesterol level and blood pressure, reduction of inflammatory problems, promotion of liver functions, reduction of obesity and regulation of appetite (Kersters et al., 2006; Malbasa et al., 2011).

Little is known about its microbial constituent. Few works have dealt with AAB involved in kombucha fermentation. Major publications focalized on *Gluconoacetobacter xylinus* that synthetizes a floating cellulose/acetan network in which the embedded cells benefit from the close contact to the oxygen containing atmosphere (De Ley et al., 1984). Moreover, yeast species belonging to the genera *Zygosaccaromyces, Pichia, Brettanomyces, Schizosaccharomyces, Saccharomycodes, Torulaspora* and *Candida* were identified in different kombucha cultures (Sievers et al 1995). The lack of knowledge in part is due to difficulties in accessing and sampling such alcoholic/acetic niches but also in isolating and maintaining recalcitrant AAB (Mamlouk and Gullo, 2012).

Components	Typical value for kombucha tea (g/L)
Acetic acid *	4.69-8.0 g/L
Ethanol	5.5 g/L

Glucuronic acid	0.0026 – 1.71 g/L
Gluconic acid	39 g/L
Glucose	12-179.5 g/L
Fructose	5.40-76.9 g/L
Remained sucrose	2.09-192.8 g/L
* Higher acetic acid content can be reached	

Table 1. Average values of components in Kombucha tea. (Jayabalan et al. 2014).

Even the dairy industry was called upon to meet consumers' needs by marketing healthy beverages such as drinkable yogurts, special milks and kefir. On this basis, our primary concern was the main byproduct of the dairy industry: the cheese whey, which is the liquid remaining after milk has been curdled. Every year a large quantity of cheese whey is produced causing many disposal problems. The major constituent of cheese whey is lactose (Table 2), contributing to the high BOD and COD content. Because of this, it is considered high-polluting agents and their disposal is still a problem in dairy sector (Carvalho et al., 2013; Lustrato et al., 2013).

For this reason, attempts have been made to qualify this liquid also for human consumption. Although black tea is the substrate used preferably for fermentation by kombucha, other liquid foods have already been tested as alternative substrates with fairly good results as reported in the study by Malbaša (2004).
The purpose of the present study arose from the need to valorize the cheese whey as a waste byproduct, together with the fulfilment of the current market requests concerning beverage with functional healthy benefits; or innovative condiment with unusual flavor profile.

Whey in fact is having a moment in top restaurant kitchens, where it's being used for many preparation from sauces and dressings to tenderizing meat and fish and even for braising root vegetables.

Artificial tenderization by acid marinating, the soaking of meat in acid solution, is a commonly used culinary technique (Aktas et al., 2003).

This work consisted in investigating the cheese whey as raw material for the production of a fermented beverage, or condiment, applying a series of progressive static fermentations.

Components	Typical value for cheese whey
Lactose (45 - 50 g/L)	45 – 50 g/L
Soluble proteins (6 g/L - 8 g/L)	6 - 8 g/L
Lipids (4 - 5 g/L)	4 – 5 g/L
Mineral salts	8 - 10% of dried extract; NaCl and KCl more than 50%
Others lactic and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins	

Table 2. Typical cheese whey composition. (Siso 1996; Panesar et al. 2007)

Another dairy product, such as milk, can be suitable as fermentative substrate for production of Kefir, another fermented beverage which is becoming more popular for its health properties.

Kefir is an acid-alcoholic fermented milk, originally made in the Balkans, Eastern Europe and the Caucasus. It is traditionally produced by inoculating milk with grains of Kefir. Some typical components' values of kefir are reported in Table 3. The industrial manufacture of Kefir using grains as the starter culture is very difficult due to the complexity of their microbiological composition, which varies widely depending on the origin of the grains and conditions of storage and handling (Kandler and Kunath, 1983; La Rivie`re et al., 1967; Ottogalli et al., 1973; Zourari and Anifantakis, 1988). At the same time, it is difficult to maintain the stability of the microbiological composition of the Kefir grains over time, i.e., during the production of successive batches (Marshall and Cole, 1985).

Components	Typical value for kefir
Milk protein	2.7 g/L
Milk fat	<10 g/L
Tritatable acidity (Lactic Acid)	>0.6 g/L
Ethanol	2.5 g/L

Table 3. Average values of components in kefir. (Simova et al., 2002).

Therefore, the aim of this research is to evaluate the use of cheese whey in combined fermentations with kombucha tea, kefir and a selected strain of AAB in order to obtain a non-alcoholic beverage or condiment with low level of acidity.

The present work was divided into three phases: kombucha tea preparation, cheese whey inoculation and fermentation, adoption of various strategies to reach the desired values of the fermentation main parameters (titratable acidity, pH, ethanol). We conducted several tests with different combinations of cheese whey, kombucha tea and kefir (a fermented beverage containing yeasts and lactic acid bacteria). A following test concerned the use of a starter acetic culture by K2G30 strain which was previously selected for its ability to produce acetic acid and bacterial cellulose (Mamlouk 2012; Gullo et al., 2019). The methodological approach used in this study was high adaptable in obtaining trials at different concentrations of metabolites of interest, especially organic acids and ethanol. Indeed, it enables the biotechnological attainment of several fermented non-alcoholic beverages or condiment, starting with the selected raw materials.

4.2 Materials and method

4.2.1 Experimental plan

The present work was divided into three main steps:

- selection, preparation and characterization of raw material through analytical determinations;
- performance of distinct fermentations;
- definition of output characteristics, through different types of analysis.

4.2.2 Cheese way selection and preparation

Cheese whey samples (5 liters) (Figure 1) were collected from a local dairy farm ("Caseificio Sociale Castellazzo" Campagnola Emilia, Reggio Emilia, Italy) one hour after the cheese-making. Temperature was superior of 24° before any fermentation process was started. Samples were stored at +4C° until use.

Fig.1. Cheese whey collected from a local dairy farm.

4.2.3 Kombucha tea preparation

Kombucha tea (Figure 3) was prepared from a commercial preparation according to the procedure reported in Sievers et al. (1995). The propagation of kombucha tea was carried out as following: one liter of water was boiled and added with 70 g of sucrose (7% w / v). Once the sucrose was completely dissolved, a tea bag (1.5 g) was added and infused for five minutes. Once the tea had cooled to room temperature, it was filtered using a vacuum pump and a 0.2 filter. The filtrate was poured under sterile conditions into two beakers (400 mL each), to which a cellulose disk (18 g) (Figure 2) from a previous kombucha was added. The remaining tea and sucrose preparation was stored in the refrigerator, to be used later as a source of nutrition for producing kombucha tea. Two beakers containing samples 1 and 2 were incubated at 28 ° C for two weeks. From each sample, 20 mL were collected every 7 days (T0, T1 and T2) and used for pH and titratable acidity measurements.

Fig. 2. Kombucha starter.

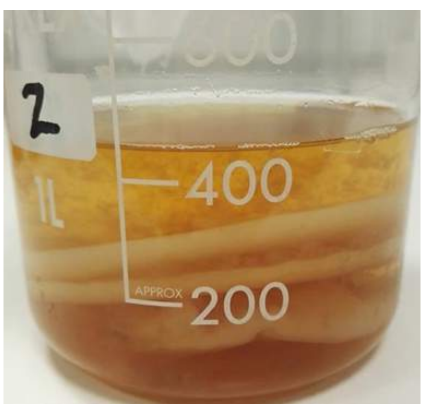

Fig. 3. Cellulose layers in Kombucha tea.

4.2.4 Kefir preparation

We obtained this fermented beverage from Kefir starter ferments, contained in a commercial powder preparation.

The powder was dissolved in a liter of partially skimmed UHT milk, leaving it to rest at room temperature for 24 hours, stirring occasionally. After the appearance of the granules (Figure 4) that signed that fermentation had taken place inside the container, kefir was placed in the refrigerator for storage.

Fig. 4. Kefir grains. (source: www.kefiralia.it)

4.2.5 Isolation, cultivation and conservation of the K2G30 strain

Strain K2G30 (species of *K. xylinus*) was previously isolated from a solid fraction of a kombucha tea sample after 6 days of fermentation and cultivated on GYC medium (Glucose Yeast Extract Calcium Carbonate). Incubation was performed at 28 °C for 4-7 days (Mamlouk, 2012).

An aliquot of the strain culture was stored at a temperature of -80 ° C by the following procedure:

- preparation of 50% glycerol solution in distilled H_20;

- 750µl of 50% glycerol solution in each vial;

- vial sterilization (121 ° C for 15 minutes);

- 750µl of culture of each strain added in each vial;

- storage at -80 ° C.

4.2.6 Whey fermentation - kombucha tea

The test was set up as follows:

- kombucha tea: 150 mL;

- whey: 150 mL;

- cellulose disk: 6 g (1/3 of a whole disk).

The test was performed in triplicate and all the steps occurred in sterility using materials previously treated in an autoclave (121 ° C / 15 minutes). The three samples were placed in a thermostat at 28 ° C for 9 days (every 3 days 20 mL were taken for each sample to perform the pH and titratable acidity analysis).

4.2.7 Whey fermentation - kefir

This test involved the use of kefir, which generally contains colonies of lactic acid bacteria and mesophilic yeasts, to be used in combination with kombucha tea and whey, according to the following quantities:

- whey: 300 mL;

- kefir: 200 mL;

- kombucha tea: 150 mL;

- cellulose disk: 9 g (half of a whole disk).

The tests were performed in duplicate. The two samples were incubated in a thermostat at 28 ° C for 6 days.

4.2.8 Acetic fermentation by starter culture of acetic acid bacteria

After 7 days from the preparation of the mixture with whey, kombucha tea and kefir, 40 mL were added to one of the two samples containing the acetic starter bacteria from the K2G30 culture, previously grown in GYC medium. The sample was incubated in a thermostat at 28 ° C for 15 days. The inoculation was carried out to favor the increase of titratable acidity in the sample.

4.2.9 Determination of pH and titratable acidity

pH and titratable acidity and were determined using the TitroLine easy © automatic titrator according to the manufacturer's instructions (CETOTEC GmbH ©).

4.2.10 Culture media for the isolation of acetic acid bacteria

GYC (Glucose Yeast Extract Calcium Carbonate)

The GYC culture medium was used for the isolation and preservation of AAB contained in the commercial preparation used to obtain kombucha tea. Before use, the medium was sterilized by autoclave at 121 ° C for 15 minutes and a pressure of 1atm. Plating was carried out by spreading a variable sample volume (0.1mL - 1mL).

GYC FORMULATION (g / L):

- Glucose: 50

- Yeast extract: 10

- $CaCO_3$: 20

- Agar: 10

The growth of AAB on GYC medium is visible from the formation of a clarification halo around colonies, due to the resolubilization of $CaCO_3$, which reacts with the acid produced by the bacteria themselves according to the reaction:

$2CH_3COOH + CaCO_3 \rightarrow H_2O + Ca^{2+} + 2CH_3COO^- + CO_2$

The plates were incubated at 28 ° C for 7 days.

4.2.11 Culture media for microbiological cheese whey characterization

1. PCA (PLATE COUNT AGAR)

FORMULATION (g / L):

- Triptone: 5

- Yeast extract: 2.5

- Glucose: 1

- Agar: 15

MEDIUM PREPARATION: suspend 23.5 g of powder in 1000 mL of cold distilled water. Bring to the boil with stirring and autoclave at 121 ° C for 15 minutes.

DESCRIPTION: this medium is used for the total count of facultative heterotrophic aerobic and anaerobic bacteria. The plates were incubated at 35 ° C for 5 days.

2. MALT EXTRACT AGAR BASE

FORMULATION (g / L)

- Malt extract: 30

- Mycological peptone: 5

- Agar: 15

MEDIUM PREPARATION: suspend 35.6 g of Malt Extract Agar in 1000 mL of cold distilled water. Bring to a boil, stir it in test tubes or bottles and autoclave at 115 ° C for 15 minutes.

DESCRIPTION: Malt Extract Agar is used for the isolation and counting of yeasts and molds from different materials. It is a medium rich in carbohydrates, which has the nutritional requirements necessary for the development of yeast-like and filamentous fungi. The selectivity of this medium is determined by the low pH value, as it inhibits most of the contaminating bacterial flora.

The plates were incubated at 25 ° C for 5 days.

3. VRBA (VIOLET RED BILE AGAR)

FORMULATION (g / l)

- Peptone: 7

- Yeast extract: 3

- Bile salt n. 3: 1.5

- Lactose: 10

- Sodium chloride: 5

- Neutral red: 0.030

- Violet crystal: 0.002

- Agar: 15

MEDIUM PREPARATION: Suspend 41.5 g of powder in 1000 ml of cold distilled water. Bring to a boil under stirring; cool in a water bath at about 45 ° C and transfer to inoculated Petri dishes.

DESCRIPTION: VRBA is a selective and differential medium indicated for the isolation and counting of coliforms in food, milk and dairy products. VRBA contains bile salts n. 3 and violet crystals that inhibit the growth of Gram positive bacteria; the neutral red allows to distinguish the fermenting lactose microorganisms from the non-fermenting lactose ones. The fermentation of lactose causes an acidification of the medium with a consequent change in the indicator towards red-violet and precipitation of bile salts.

The plates were incubated at 35 ° C for 5 days.

4.2.12 Bacterial count

For the estimation of the microbial load present in cheese whey, the samples were aliquoted using the method of serial dilutions in test tubes in order to create scalar decimal dilutions. A known volume of sample (1mL) was uniformly seeded by spatulation on the PCA and Malt Extract Agar Base media, instead of being included in the VRBA medium. All the microbial counting tests were performed in triplicate and the results were expressed by calculating the average between them.

Estimated bacterial load (U.F.C / mL) = No. of colonies per plate * Dilution factor

4.2.13 Ethanol quantification with enzymatic kit

Ethanol was measured enzymatically using the kit (K-EtOH) from Megazyme, following the procedures and methods provided by the manufacturer and reported in the manual. The ethanol concentration was analyzed in the final sample of the drink obtained. All enzymatic kits are based on the difference in ultraviolet absorption spectra between the oxidized and reduced form of the coenzymes (NAD + / NADH). It is therefore possible to measure the conversion of the coenzyme from one form to another during enzymatic tests by measuring the UV absorbance at 340 nm by spectrophotometer.

4.2.14 Chemical-physical whey analysis

4.2.14.1 Determination of moisture content

The total humidity was determined by drying in a stove. The analysis allows to calculate both the total water content and at the same time to report the analytical data, measured on the wet sample, to the dry substance.

Method

The empty tares in which the sample is subsequently measured were placed in a preheated stove at 105 ° C for 30 min, and allowed to cool (henceforth handled only with steel tongs). The empty tares were weighed with the use of an analytical balance, and after having written down the value, 5 g of sample were weighed directly inside the tare. The whole was transferred back to the oven at 105 ° C for 3 h. After the necessary time has elapsed and after having allowed to cool, the tare containing the dried sample was weighed (Figure 5).

Calculation

The percentage x of humidity is obtained according to the formula:

$$x = \frac{m_1 + m_c - m_2}{m_c} \; X \; 100$$

Where:

m1 = mass of the empty tare [g]

mc = mass of the sample [g]

m2 = total tare mass and dried sample [g]

Fig. 5. Tares with dried sample.

4.2.14.2 Determination of the ash content

The whey ashes represent all the mineral substances present in it. To carry out the analysis the principle of evaporation of the liquid fraction of the sample is exploited, determining the content of ash left through simple weighing.

Method

Through the use of an analytical balance, 10 g of sample were weighed directly inside a known tare weight porcelain (previously kept in an oven at 105 ° C for 30 min).

The tare containing the sample was placed in an oven at 105 ° C for 1 h and subsequently transferred to a muffle at 520 ° C for 2 h. After allowing to cool, the tare containing the sample was weighed at the end of the drying process.

Calculation

The weight decrease represents the ash content of 10 g of serum, therefore the x percentage value of ashes present is obtained according to the formula:

$$x = \frac{m_2 - m_1}{m_c} X\ 100$$

Where:

m2 = total tare mass and dried sample [g]

m1 = mass of the single tare [g]

mc = mass of serum sample [g]

4.2.14.3 Determination of lipid content

The percentage of lipids present in the serum sample was determined using the Soxhlet method. This is a hot extraction technique that exploits the ability of a solvent (petroleum ether) to solubilize the lipids and separate them from the other components.

The Soxhlet extractor (Figure 6) consists essentially of three parts:

a) a flask containing the solvent;

b) the actual extractor;

c) reflux condenser, water cooled.

Moreover, in the extractor a side arm allows the passage of solvent vapors to the condenser, while the siphon allows the periodic emptying of the extractor. The material containing the compound to be extracted is placed in a thimble, made of porous cellulose, which is inserted into the main chamber of the extractor.

Method

- 2.5 g of sample were weighed, placed in a special cellulose thimble between two layers of cotton that prevent the material from escaping during extraction. With the use of an analytical balance the empty balloon was weighed.

- The thimble was inserted in the appropriate chamber of the extractor and the flask containing the solvent was placed in the lower part of the appliance.

- After assembling the equipment, the balloon containing the ether was heated.

- At the end of the washings with the solvent, the flask with the extracted material was recovered and weighed.

Operation

The vapors of the heated solvent reach the refrigerant and flow back into the thimble, which is slowly filled with the hot solvent. When the thimble chamber is almost full, it is automatically emptied from the side trap. The solvent, enriched with the compound to be separated, falls into the flask. This cycle of washes is repeated several times, even days, until complete solvent evaporation. Eventually the extracted substances concentrate in the distillation flask.

Calculation

The percentage x of the lipid content was calculated as follows:

$$x = \frac{m_2 - m_1}{m_c} \; X \; 100$$

Where:

m2 = mass of the flask containing the extracted lipids [g]

m1 = mass of the empty balloon [g]

mc = mass of the sample [g]

Fig. 6. Soxhlet extractor.

4.2.14.4 Determination of lipid content

The whey protein content was determined following the procedure provided by the Kjedahl method. Through this method, which involves digestion by boiling, the nitrogen content is measured, which is a direct index of the protein content since it is believed that most of the nitrogen contained in foods derives from proteins. There are specific conversion factors for each type of food which, given the calculated nitrogen value, provide the percentage of proteins present.

Method

- 15 g of K_2SO_4 were added inside a digestion tube, which acts as an antifoam and raises the boiling temperature of the solution and 1 mL of $CuSO_4$ which catalyzes the hydrolysis of proteins.

- 5 g of sample were weighed, heated to a T of about 38 ° C and added to the digestion tube.

- 25 mL of H_2SO_4 were added, leading to high T (300 ° C) through a heating plate. The procedure takes about 1.5 hours.

- Thanks to this boiling process the proteins are completely degraded and the individual atoms (H_2, C, N, O_2) are recombined, giving rise to new molecules (CO_2, H_2O, NH_3). Molecular H_2, molecular O_2 and CO_2 evaporate and water and ammonia remain, which would also be subject to evaporation as a gas, but thanks to the copper sulphate added at the beginning it is trapped as ammonium sulfate.

- At the end of digestion 75 mL of 50% NaOH were added which caused a displacement reaction then sodium sulfate and liquid ammonia (NH_4OH) were reformed.

- Ammonia was distilled, collected in boric acid (H_3BO_3) and titrated with a solution of HCl. What is actually titrated is the OH present in the NH_4OH molecule. Quantification of OH is a direct way to quantify ammonia as the two species are present in equimolar amounts.

Calculation

At the end of the titration the calculation of the nitrogen content is performed using the following formulas:

g (N) = moles of N x 14 $\quad\quad$ $\% N = \frac{g\ (N)}{g\ (campione)} \times 100$

The conversion factor used for whey is 6.37 so: % proteins =% N x 6.37.

4.3 Results and discussion

4.3.1 Raw material selection criteria

In this work, an unconventional raw material (cheese whey) was considered in order to assess its suitability for a series of different progressive static fermentations, aimed at obtaining non-alcoholic beverages or low acidity condiments.

The criteria adopted for selection of the raw material and the fermentative cultures (Table 4), responds to a series of different and emerging need assessed during the market analysis.

Cheese Whey	Kombucha tea	Kefir
• Main by-product of the dairy industry • Use of leftover raw material • Sustainability as added value • Unconventional fermentative substrate	• Healthy drink as output • Non-alcoholic beverage • Fermented product from tea • Unconventional symbiotic cultures	• Healthy drink output • Non-alcoholic beverage • Unconventional symbiotic cultures

Table 4. Selection criteria of raw material and fermentative cultures.

The combination of unconventional raw material to which unconventional fermentative culture were applied, lead us to design an innovative non-alcoholic beverage or low acidity condiment, having a combination of value added.

4.3.1.1 Cheese whey

Due to the need to recover cheese whey as a by-product of the dairy industry and to valorize it due to its nutritional properties, it was therefore the subject of our study. The raw material was analyzed from different points of view, to assess which parameters could have affected our experiment. On the day of collection at the dairy, the cheese whey (Figure 7) was transferred to the laboratory under refrigeration conditions to perform microbiological tests; other aliquots were instead frozen for subsequent analysis. Three different types of isolation media were used to promote the growth of different microorganisms in each one. The PCA medium was used for the

isolation of aerobic and facultative anaerobic bacteria, the Malt Extract Agar Base medium for isolating yeasts and molds, the VRBA medium instead for coliform counting. After 5 days of incubation at the respective temperatures, direct plate counting was performed. The results (Table 5) showed microbial growth up to the third dilution (1: 3) of the starting sample in the selective PCA medium for aerobic and facultative anaerobic bacteria. In the other two media, VRBA and Malt Extract Agar Base, the microbial growth was rather limited, already from the first dilution (1: 1) of the whey sample; there were therefore no significant quantities of yeasts, molds and coliforms. In general, these results show a relatively low microbial load, this is probably due to the cooking phase (55 ° C for 20 minutes) and subsequent resting of the whey in the boiler, which eliminates the flora more sensitive to high temperatures, selecting the thermophilic one. These results are in line with those reported in the bibliography, which show how the cooked whey microflora consists predominantly of thermophilic lactic acid bacteria, emphasizing the disappearance of other undesired microorganisms.

Fig. 7. Cheese whey collecting site.

Media	CFU/mL
PCA	N.C. $106.6 \times 10 \pm 41.13 \times 10$ $25 \times 10^2 \pm 5.03 \times 10^2$ $1.66 \times 10^3 \pm 1.15 \times 10^3$

MALT EXTRACT AGAR BASE	15 ± 5.86 $2 \times 10 \pm 1 \times 10$
VRBA	3 ± 1 $1 \times 10 \pm 0.57 \times 10$

Table 5. Microbial counting on PCA, Malt extract agar and VRBA media.

The average of the measurements made on the pH of the whey sample provided a value of 4,43± 0,06. As for the moisture and ash content, the whey analyzed by us was in compliance with the data reported in the literature (Mawson 1993), with a value of 93.05% for the water content and 0.52% for the content of ashes (Table 6). The fat content calculated with the Soxhlet method was slightly higher than the average of the data reported in the bibliography (Pescuma et al., 2008) with an average of 0.45% among the three samples. The protein content measured with the Kjedahl method was also slightly higher than the tabulated data, with a value of 1.1%, this minimum difference could be due to the presence of foreign particles inside the sample. Although it was not possible to measure it directly, it is believed that also the lactose content of the sample corresponded to the average values reported in the literature (0.5%). However, it is always necessary to consider the variability factors of the whey composition, such as the quality of the milk and the cheese production technique (coagulation temperature and curd cutting time).

pH	HUMIDITY	HASHES	FAT	PROTEIN
4,43	93,05%	0,52%	0,45%	1,1%

Table 6. Cheese whey parameters detected.

4.3.1.2 Kombucha tea

In this part of the study, we focused on obtaining traditional kombucha tea in the laboratory, using a standard inoculum matrix consisting of black tea and sucrose. For this purpose, a commercial kombucha tea aliquot was cultivated in order to maintain the vitality of the microorganism present (yeasts and acetic bacteria) and to obtain a sufficient volume for subsequent tests.

From the sample aliquots collected during the entire incubation period, a slight increase in titratable acidity (Figure 9) was found, accompanied by the formation of different layers of

bacterial cellulose, both due to the activity of the AAB present (Figure 8). As reported in Sievers and coauthors (1995), the measured pH would have to undergo a decrease reaching values around 2.5, consequent to the transformation of the ethanol produced by the yeasts into acetic acid and to the presence of gluconic acid. In our samples, on the other hand, ethanol was present in not enough quantity to be detected through the use of the enzymatic kit (see materials and methods) and this did not allow the achievement of the acetic acid values reported after 20 days of fermentation (6 g /L). This different trend of fermentation could be due to the presence of yeasts of different species within the commercial preparation compared to those reported by Sievers (1995). In fact, in this study, yeasts of the genus *Zygosaccharomyces* were isolated from kombucha tea, which after having hydrolyzed sucrose into glucose and fructose, quickly converted fructose into ethanol and CO_2. Since fructose is metabolized before glucose in the fermentation of kombucha tea, the yeast cells have a decisive influence during the production of both metabolites. Furthermore, the kombucha tea of the present study was analyzed from the microbiological point of view, sowing 1 mL of sample in GYC and YPD medium as reported in materials and methods. After the incubation period, the plates had a charge of 2.4 x 105 CFU / mL of sample in the GYC medium and 5.6 x 104 CFU / mL of sample in the YPD medium, respectively. These values are in agreement with the data reported in the bibliography (Kanuric et al., 2018) for the microbiological charge of kombucha tea and have highlighted both the presence of yeasts and AAB.

Time (days)	Sample n.	pH	Titratable Acidity (g/L)
0	1	2.98	0.62
	2	2.97	0.62
7	1	2.86	0.86
	2	2.87	0.86
14	1	2.73	1.22
	2	2.75	1.11
21	1	3.0	1.22
	2	3.03	0.62

Table 7. pH and titratable acidity values of Kombucha Tea.

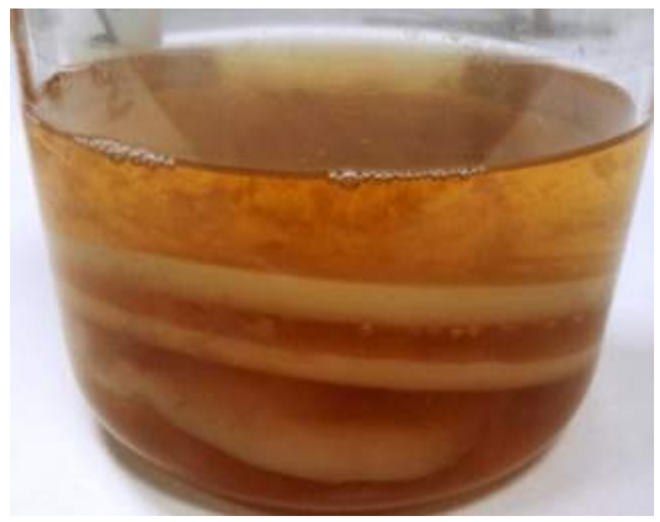

Fig. 8. Layers of bacterial cellulose in kombucha tea.

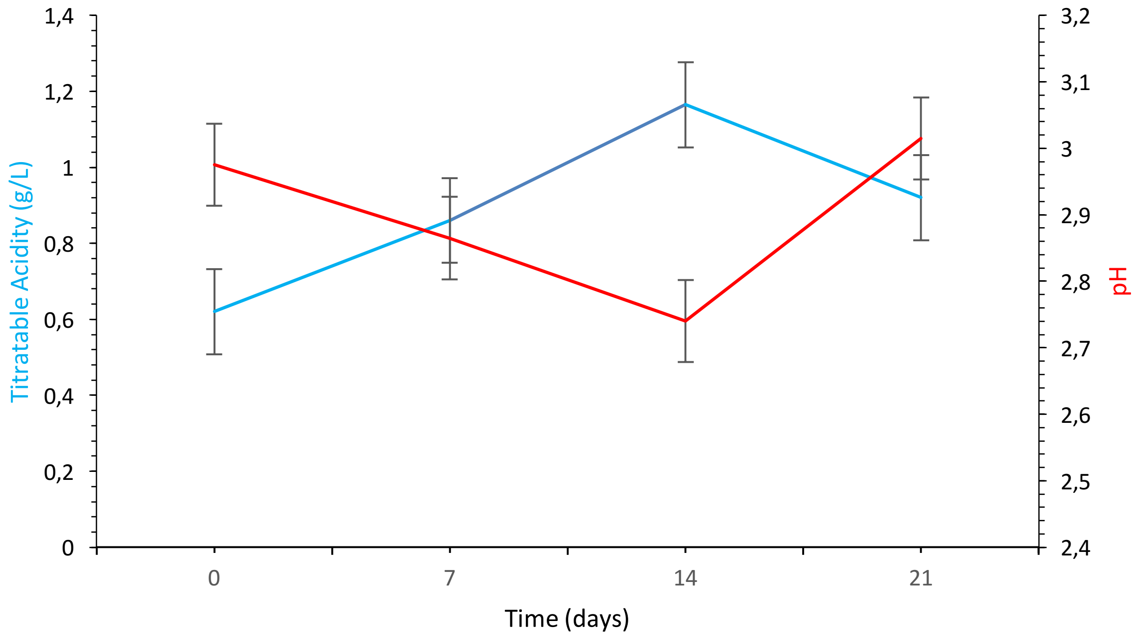

Fig. 9. pH and titratable acidity trends in Kombucha Tea.

4.3.1.3 Kefir

The kefir was obtained from a freeze-dried commercial mixture added to a Liter of partially skimmed UHT milk. The acidity after 24 hours of fermentation was 0.7% v/v. This value is in accordance with that reported by the Codex Alimentarius (min. 0.6%) for fermented milks. A

sample of kefir was analyzed using an optical microscope, and we detected the presence of cocci-shaped bacteria and bacilli and yeasts. As reported in the bibliography (Irigoyen et al., 2003), the microbiology of kefir is complex and quite variable, lactic acid bacteria are usually present in greater concentration followed by yeasts and AAB. For our study, we selected kefir for its lactose fermenting yeast content, high enough to increase the production of ethanol in the sample.

4.4 Fermentation tests

4.4.1 First test: kombucha tea/cheese whey

The first test was carried out using the cheese whey as fermentative substrate with addition of kombucha cultures in a 1: 1 ratio (Figures 10). The samples were incubated at 28 ° C for 9 days. The collected samples provided the results shown in Table 8.
Observing the titratable acidity levels (Table 8, Figure 11), we can assume that the yeasts contained in kombucha did not have a fermentative activity on lactose, in order to produce a sufficient amount of ethanol, which is subsequence fermented by AAB converting it into acetic acid.

Lactose is a disaccharide formed by galactose and glucose and is chemically defined as O-ß-D-galactopyranosyl-(1-4)-ßD-glucose, is the main carbon source for starter cultures of lactic acid bacteria used in the production of fermented dairy products. Lactic acid bacteria ferment lactose present in the substrate mainly through two ways: homofermentative metabolism (with almost exclusive production of lactic acid) or heterofermentative metabolism (production of ethanol and CO_2 in addition to lactic acid). As far as kinetics and lactose conversion methods are concerned during the fermentation of milk by an inoculum of kombucha tea, there are still no data available in the literature. However, several studies assessed the suitability of the fermentation operated by kombucha, to a raw material containing lactose (Iličić et al. 2012). Moreover other studies report fermentation activity of lactose from low-fat milk inoculated with kombucha starter. Milanovič et al. (2012) used kombucha on different types of tea, combining them with a probiotic starter in fermented dairy products; Vukič et al. (2014) studied the effect of a kombucha starter inoculation on rheological properties, structure and microstructure, and protein fractions at different stages of milk fermentation.

Fig. 10. Cheese whey/Kombucha tea sample.

TIME (days)	Sample N.	pH	Titratable acidity (g/L)
0	1	3.51	0.62
	2	3.55	0.63
	3	3.53	0.62
3	1	3.42	0.92
	2	3.42	0.92
	3	3.40	0.92
6	1	3.28	0.92
	2	3.27	1.04
	3	3.23	0.98
9	1	3.23	1.24
	2	3.26	1.36
	3	3.17	1.32

Table 8. pH and titratable acidity values of the first test.

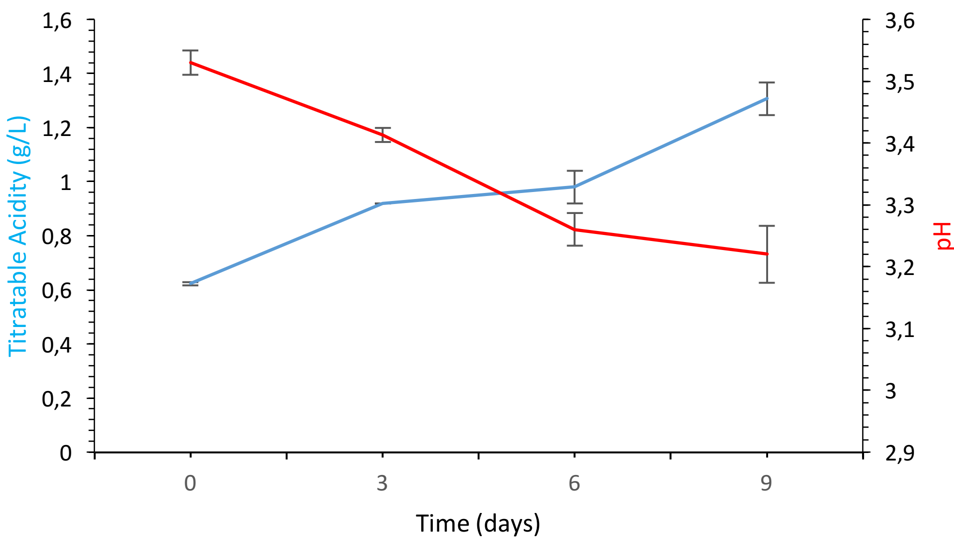

Fig. 11. Trend of pH and titratable acidity values in kombucha tea and whey samples.

4.4.2 Second Test: kombucha tea, cheese whey and kefir

The second test was performed adding kefir culture to the previous batch composed by kombucha tea and whey (Figure 12).

This test was performed in order to:

- to enrich the substrate with kefir components;

- to exploit the presence of the *Kluyveromyces* yeasts present in kefir capable of fermenting lactose in order to eventually increase the ethanol content available for AAB growth.

In this case, as we can see from Table 9 and Figure 13, the titratable acidity values increased slightly during the observation period (7 days). It is important to point out that lactose-fermenting yeasts (e.g. *Kluyveromyces lactis*, *Kluyveromices marxianus*) and non-fermenting yeasts (*Saccharomyces cerevisiae*) are present in kefir grains. This factor could have influenced the results obtained in this experiment, since the percentage of one or the other was not known within the lyophilized preparation we used.

Fig. 12. Samples of mixed Kombucha tea, whey and Kefir.

TIME (days)	sample N.	pH	Titratable acidity (g/L)
0	1 2	3.49 3.44	1.24 1.24
3	1 2	3.39 3.41	1.54 1.53
6	1 2	3.36 3.43	1.79 1.41

Table 9. pH and titratable acidity values of the second test.

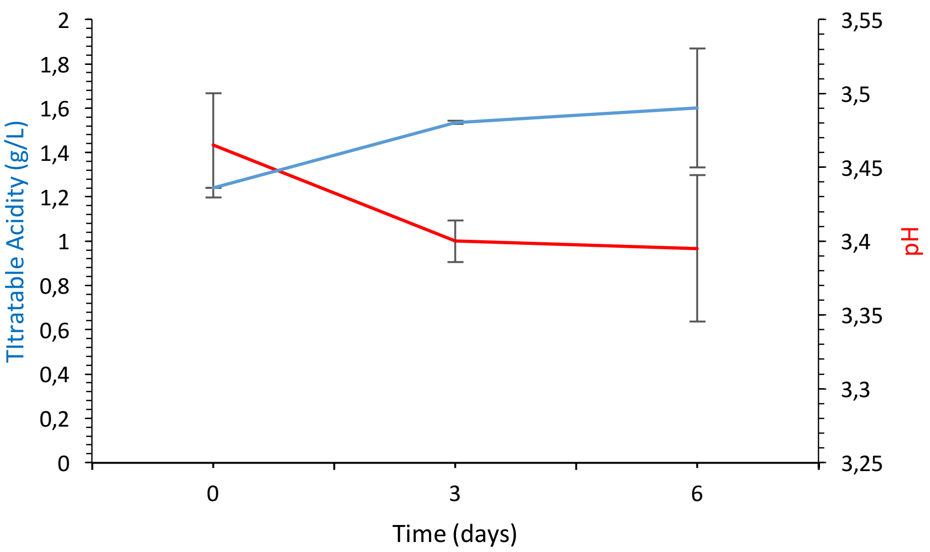

Fig. 13. Trend of pH and titratable acidity values in mixed kombucha tea, whey and kefir samples.

4.4.2.1 Third test: use of AAB starter culture

The raw materials tested, kombucha tea, whey and kefir showed an attitude to be used as substrates for obtaining innovative condiments or non-alcoholic fermented beverages. Particularly, the whey used, given the low microbial load detected, is suitable for this purpose, as well as being a good source of proteins, vitamins and minerals with reduced fat content. These innovative beverages are also naturally enriched by the active microflora present in kombucha tea and kefir, without the use of additives. Therefore, the application of selected starter strains can significantly improve the quality of whey, thanks to the properties of kombucha tea and kefir, which increase the health component of the final product, in addition to amplifying the sensory characteristics. As reported in the literature (Greenwalt et al., 1998; Jayabalan et al., 2007) in fact, there are numerous benefits that the consumption of kombucha tea brings to health, such as the consumption of kefir, defined as a real functional food and probiotic. It is believed that these health effects are the result of a complex synergistic mechanism between inoculated microorganisms (in our case a complex microbial flora consisting of yeasts, acetic bacteria and lactic bacteria), the characteristics of the raw material and the fermentation substrates. Following in-depth research into the non-alcoholic fermented beverages on the market, it was found that the typical titratable acidity values of these kind of drinks are generally 2-3.5%. The same level of acidity and pH can be found in condiments and seasonings used for marinating, which are widely used by consumers to improve meat tenderness and flavor (Whipple and Koohmaraie, 1993).

Being a mechanical property, tenderness is related to final pH, postmortem temperature, sarcomere length, connective tissue content and enzymatic proteolysis of myofibril proteins (particularly troponin and desmin).

Therefore, in the present work, it was chosen to perform a fermentation mixing cheese whey, kombucha tea, kefir with a selected starter culture obtained from the K2G30 strain.
This strain was chosen for its ability to produce acetic acid from ethanol and bacterial cellulose as the main exopolysaccharide. The characteristics of the strains are shown in Table 10.
In kombucha tea where the prevailing sugar is sucrose, which cannot cross the bacterial cell membrane, the production of bacterial cellulose takes place starting from the hydrolysis of glucose and fructose. Thus, in this way the two sugars can be actively transported inside the cells through glucose and fructose permeases and, following a series enzymatic reaction, converted into bacterial cellulose.
Thanks to the activity of the inoculated strain, the titratable acidity value has increased, as can be seen in Table 11, over a two-week observation time (Figure 14).
The residual ethanol value measured with an enzymatic kit was found to be: 0,23 $\pm$ 0,003 g/L.
The results obtained are comparable with the characteristic parameters of other non-alcoholic fermented beverages, without added sugars and aromas, both as regards the pH and titratable acidity and ethanol values. Furthermore, these results could be compatible with the characteristics of a product defined as a condiment, for which there are no legal limits to be respected with regard to titratable acidity and, at the same time, match the ideal requirement for marinating process.
In fact, according to Giudici et al. (2015), condiments cannot be defined as vinegars due to low acidity, composition and intended use, although they often resemble vinegars very much. There are no limits regarding their formulation, they can contain any type of additive, both artificial and natural.
The sample obtained in this study presents all the characteristics of an eco-sustainable product, enhancing whey and reducing its environmental impact, as well as having a high health potential or functional attitude in food preparation.

K2G30 strain	
Growth on solid media	Mainly by soft cellulosic biofilm
Growth on broth	Visible cellulosic layers
Gram reaction	-
Catalase reaction	+
Cell shape	rod
Calcium carbonate oxidation	+
acetic acid production	+
Gluconic acid production	+
Cellulose production	+
Species	*Komagataeibacter xylinus*

Table 10. K2G30 strain features (Data from Gullo et al., 2019)

TIME (days)	pH	TITRATABLE ACIDITY (g/L)
0	3.33 ± 0.02	1.55 ± 0.03
3	3.42 ± 0.015	1.86 ± 0.05
6	3.42 ± 0.02	2.03 ± 0.04
9	3.26 ± 0.025	2.16 ± 0.015
12	3.29 ± 0.01	2.19 ± 002
15	3.28 ± 0.02	2.21 ± 0.01

Table 11. pH and titratable acidity values of fermentation trials.

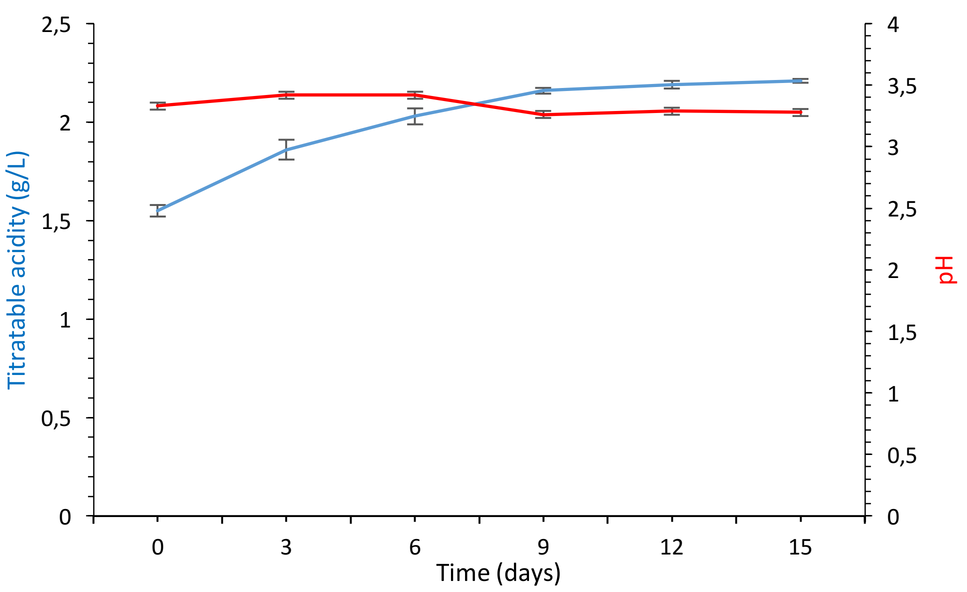

Fig. 14. Trend of pH and titratable acidity values of the fermentation trials.

4.5 Conclusions

The versatility of the fermentation process employed and its positive impact on the quality of the food itself, demonstrates the potential of microorganisms in the industrial food processing sector. In particular, the fermentation processes such as the acetic one exploit the action of specific microorganisms to increase the shelf life and the nutritional benefits of fresh foods with reduced shelf-life, such as cheese whey. Not only acids and alcohol exert a protective action against the substrate, but also other metabolites resulting from the fermentation process. Furthermore, the use of innovative raw materials, with peculiar characteristics, can be translated into obtaining high added value products, currently very requested by the food industry market. The different possible combinations of starting materials and fermentation methods allow to obtain a variable composition, in response to highly differentiated needs of consumers.

The range of variability in which the finished products can be defined defines the type of drink. As with any other fermented product, the production limits could be the standardization of the process and the stability of the finished product, even if the companies are trying more and more to remedy these technological difficulties.

Finally, the strategy applied in this study is the starting point for obtaining vinegar based product using unconventional raw materials and applying a static fermentation. This type of product could be an alternative to simple whey vinegar, enriched by the typical flavor given by kombucha tea.

4.6 References

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Chapter 5

ITALIAN RICE VINEGAR MADE WITH ARTISANAL METHOD AND SELECTED CULTURES

CHAPTER 5. ITALIAN RICE VINEGAR MADE WITH ARTISANAL METHOD AND SELECTED CULTURES

5.1 Introduction

Cereals vinegars are widely produced in Asiatic countries from ancient time. They can be obtained from several cereals such as barley, wheat, millet, sorghum and rice. In China, Japan and other Asian countries, an age-old and traditional process, known as solid-state fermentation (SSF), is largely used to produce vinegars from cereals at small-scale.

The basic process steps of these kinds of vinegar are: (1) crushing and steaming of cereals;

(2) addition of water and Qu or Koji (respectively in Chinese and Japanese) that is a specific cereal preparation containing molds, yeasts and bacteria; (3) alcoholic fermentation; (4) acetic acid oxidation during which wheat bran and rice (or other cereals) hull are mixed with old Pei (acetic acid fermented product from last batch as seed vinegar) (Wu et al 2009).

Chinese vinegars are used as a traditional condiment derived from grains and are frequently used in cooking and for production of foods. Shanxi old mature vinegar is the most famous vinegar in northern China, where this vinegar is produced using sorghum as its main material for the fermentation process (Ho et al., 2017).

Rice (*Oryza sativa L.*) is the premier food crop in the world, being a major staple food for more than half of the world’s population. In Eastern countries rice vinegar has overcome traditional use as a condiment, reaching a new popularity as base of beverages with functional properties (Chen and Chen, 2009; Chen et al., 2012; Fan et al., 2009).

The rice vinegars komesu and kurosu are produced from polished and unpolished rice, respectively, by the same process (saccharification of rice, alcohol fermentation, and oxidation of ethanol to acetic acid). Both of these vinegars are traditional seasonings that have long been used in Japan, China, and Asian countries. Komesu is colorless and has a plain taste and thus is used for sushi cooking, while kurosu is black and contains more amino acids and vitamins than komesu and thus is used as a healthy drink (Nanda et al 2001).

In Italy, there is rather a more rooted tradition of growing and consuming rice varieties with white kernels which are very appreciated by consumers worldwide thanks to their quality traits.

In terms of foreign exchange, rice (*Oryza sativa L.*) is one of the most economically important crops in Italy, with a cultivation area that is mostly located in the Po Valley in the provinces of Alessandria, Ferrara, Novara, Pavia, and Vercelli, and to some extent in Sardinia, in the southern part of the Country (Tesio ed al 2014, Fasola and Ruiz 1996). Altogether these areas are actually the largest rice producers in the European Union, accounting for almost 50% of European rice production with 1.44 millions of tons (Ente Nazionale Risi).

Italian rice production, which generates annual turnover amounting to EUR 1 billion, was suffering by the sharp increase in duty-free rice imports from South-East Asia, especially from least developed countries such as Cambodia (249 000 tons, up 822%) and Myanmar/Burma (62 000 tons, up 400%). Because Asian imports have been increasing, the areas under rice in Italy are being drastically reduced (by 50% in the case of the *indica* variety). Italian rice is being subjected to unfair competition from Asian rice, for which the production costs are much lower and which does not conform to environmental and social sustainability standards (Parliamentary questions, February 7th 2018).

Italian producers and trade associations have been calling on the EU to take specific measures to protect rice.

Following the market and competitive analysis performed in Chapter 1, we found Italian rice as a suitable raw material for the two innovative driver applications.

As answer to the assessed emergent needs, rice as raw material for vinegar, can fit the consumer's request for:

- Organoleptic and sensory characteristics. Even though rice vinegar is consumed in Asian countries since centuries, is not a common seasoning for European and American consumers. As we noticed for the continuous growth of apple vinegar's choice (where

Italian production account for almost 20% on European production with 2.2 millions of tons), we think that also rice can be appreciated for:

  - the outlandish flavor for westerns food cultures;
  - lower total acidity in acetic acid, compared to wine vinegar, and apple vinegar alike (usually around 5%). Moreover, differently than wine and apple vinegar, rice has a different organic acid composition that will end in a milder flavor (less astringent and less sharp);
  - it doesn't significantly alter the appearance of the food (Ho et al., 2017). For the same reason, a strictly-traditional product like Balsamic Vinegar, faced the needs to add the category of so called "white balsamic". The latter product is outside the PGI schemes and is classified as "Condiment". The request for a dramatic change in appearance was driven by the need of less visual impact on dishes.

- "health claim": rice is gluten free, differently than other cereals vinegars.

For the market approach, instead, the application of the artisanal method (static surface fermentation) can elevate the perceived value for a higher market positioning.

Rice vinegar made with submerged fermentation (industrial method) are already present on the European market. Average price is not monitored. In this study, we investigated how the same raw material used for the industrial process, could react with a different method. In this study already saccharified rice (rice syrup) was used as raw material to produce rice vinegar.

5.2 Materials and methods

5.2.1 Raw material

Rice syrup was the raw material used for acetic fermentation. It was produced by the Galletti S.n.c company (San Daniele Po, CR). The main composition (expressed as % dry substance) as declared by the producer is: long chain sugars 16; maltotriose 8; maltose 46; fructose 0; glucose 28.8.

5.2.2 pH and titratable acidity and ethanol determinations

pH and titratable acidity were measured using an automatic titrator (TitroLine®EASY) as reported in Chapter 2. Ethanol was measured by ebulliometric analysis using the Malligand device.

5.2.3 Brix degree

Soluble solids expressed as degrees Brix were determined by refractometer measures (Refractomer mod. 2WA, Alessandrini, Italy).

5.2.4 Acetic acid bacteria isolation from fermenting vinegars

Samples were collected at Acetaia San Giacomo from six fermenting vinegar showing a visible superficial biofilm and transferred to the laboratory (Table 1, Figure 1). Isolation from samples was performed at the same day of collection.

Direct isolation from samples was performed plating 1 mL of surface liquid on GEY medium (Table 2). Plates were incubated at 28 °C for a period of 4-5 days. After that colonies were picked up and purified on GEY medium.

The clear halo and the re-precipitation of calcium carbonate on plates was used as criterion to establish the AAB genus. The strains were labeled as SG (San Giacomo) plus the number of collection sample and preserved at +4 °C in GEY and -80°C (glycerol 50 % (v/v added to the GEY broth).

Sample label	Sample	Titratable acidity
35	White wine	4.44
43	White wine	4.60
40	Red wine	5.40
36	Red wine	5.40
14	Grape must	5.04
23	Grape must	5.04

Table 1. Characteristics of samples.

D-glucose	20 g/L

Yeast extract	10 g/L
Calcium carbonate ($CaCO_3$)	3 g/L
*Agar	15 g/L
Distilled water	1 L

* Agar was added for solid medium preparation

Table 2. Isolation and preserving medium composition (GEY medium/broth).

Fig. 1. Collection of different samples.

5.2.4.1 Phenotypic features of strains

Cell shape was observed after incubating cultures in GYE broth at 28 °C for 4 days using a C. Zeiss microscope apparatus (Axiolab) magnitude 100X. Gram-staining and catalase production were tested as described previously (Navarro and Komagata, 1999). Oxidation of ethanol to acetic acid and acetate assimilation was tested on EYC medium containing (per liter) ethanol 30 ml, yeast extract 10 g, calcium carbonate 20 g, agar 8 g; plates were observed after 4 days and 10 incubation at 28 °C, respectively.

5.2.5 Alcoholic fermentation of rice syryp

The alcoholic fermentation (Figure 2) was conducted on diluted rice syrup (ratio rice syrup:water 2:3) using a commercial yeast strain of *Saccharomyces cerevisiae*. Samples were incubated at 25 °C until reaching 7.5% of ethanol content.

Fig. 2. Rice syrup during alcoholic fermentation.

5.2.6 Laboratory scaling up of bacteria cultures in rice syrup

Bacterial strains were cultivated in GY broth (glucose 10%; yeast extract 1%, Oxoid Ltd, Basingstoke, UK) for 5 days at + 28°C. The cultures when then transferred in flasks containing 200 mL of fermented rice syrup (ethanol 6,5% v/v). After reaching suitable titratable acidity (≥4.0% v/v) values, samples were transferred at San Giacomo company for a further scaling up.

5.2.7 Factory scaling up of bacteria cultures in rice syrup

Bacterial strains were cultivated on GY broth (glucose 10%; yeast extract 1% (Oxoid Ltd, Basingstoke, UK) for 5 days at + 28°C, then 10 mL of culture was added to fermented rice syrup the scaling up was started from 200 mL of culture having ethanol content of 6.5% (wt/v)). Flasks were maintained at room temperature. The titratable acidity and ethanol content were

checked every 3 days. When residual ethanol was around 0.5% a further scaling up was done, according to the procedure of the laboratory scaling up.

5.3 Results and discussion

This part of the PhD project was aimed at the development of acetic fermentation from rice syrup. To this aim, rice syrup from the Galletti s.n.c company was used as raw material. The alcoholic fermentation of diluted ryce syrup was successful conducting by a commercial oenological *S. cerevisiae* yeast strain.

5.3.1 Acetic acid bacteria strains isolated from Acetaia San Giacomo

From the isolation procedure adopted in this study, colonies with the same morphology were observed. Although the colony morphology is not a robust discrimination character, it is possible to suppose that a stable cultivable microflora was established in the samples chosen for isolation. This is a common phenomen examining microbiological environments in which the selective pressure due to a stringent composition (eg. acetic acid content, pH, ethanol) allows the growth of a restricted number of different strains and/or makes cells slow growing or not cultivable in culture media (Mamlouk et al., 2011). Then to avoid isolates redundancy, a single colony from each plate was picked up and purified. The 6 isolates were named as strains: SG14, SG23, SG35, SG36, SG40 and SG43, respectively. All the strains showed the basic phenotypic traits of AAB: gram-negative, catalase positive reactions, mainly occurring in pairs or in short chains and all oxidized ethanol to acetic acid. Moreover, acetate assimilation was observed for all the culture strains by the reappearance of opacity on the bottom of the plates due to overoxidation, when incubation on EYC was extended until 10 days. The acetate assimilation is distinguishing for members of AAB providing useful information on phenotypic traits with taxonomic relevance and if evaluated over cultivation time also on the stability of traits (Gullo et al., 2012). Metabolically, acetate oxidation it is caused by increasing activity of Krebs cycle enzyme and also by increased NADH oxidase activity, as a sophisticated strategy to survive in acetate stress environment (Matsushita et al., 2004). It occurs in all the member of *Acetobacter, Gluconacetobacter* and *Komagataeibacter* genera and it is recognized as distinctive of AAB growing in acetate media, like vinegar; it is not a trait of *Gluconobacter*

species, which have not the complete enzymatic set to perform Krebs cycle (Matsushita et al., 2004). From several literature reports it is known that *Acetobacter* strains are widely detected in vinegars which acidity is around 6%, including also cereals vinegar, where generally the species *A. pasteurianus* (Nanda et al., 2001; Wu et al., 2009) is described. Whereas in high acidity vinegar (acetic acid content higher than 10%) *Komagataeibacter europaeus* is the species detected at high frequency (Gullo et al., 2014).

On the basis of all these observations, strains of this study could belong to *Acetobacter* genus. At the current, a polyphasic identification work, including 16S rRNA sequencing and a further phenotypic characterization is being done to ascertain the species allocation of the 6 isolated strains.

5.3.2 Rice syrup acetic fermentation by AAB strains from Acetaia San Giacomo

Laboratory scale fermentations were conducted to the aim to evaluate the ability of SG strains to grow and produce acetic acid in rice syrup containing more than 6% of ethanol. A scaling up was conducting starting from 10 mL of culture of each strain. The following experiments were performed in 250 ml flask (incubating temperature 28 °C), adding AAB cultures (10 ml) in 200 ml of rice syrup wine (6.5% ethanol) and monitoring acetic acid and ethanol every 3 days until ethanol was equal or lower than 0.5%. The best performance in term of acetic acid production was observed for trials developed with strain strains SG14 (3.97 acetic acid) and SG35 (2.75 acetic acid), whereas slow growth and very low acetic acid content was reached for trials conducted by SG40 (0,32 acetic acid) and SG43 (0,32 acetic acid).

The two culture strains (SG14 and Sg 35) were used to develop a static prototypal acetic fermentation at San Giacomo company were the fermentation was conducting for 60 days providing an acidity of 6.1% v/v (Figure 3).

In Figure 4 the fermentation trend is shown.

Fig. 3. Prototypal acetic fermentation developed with two culture strains.

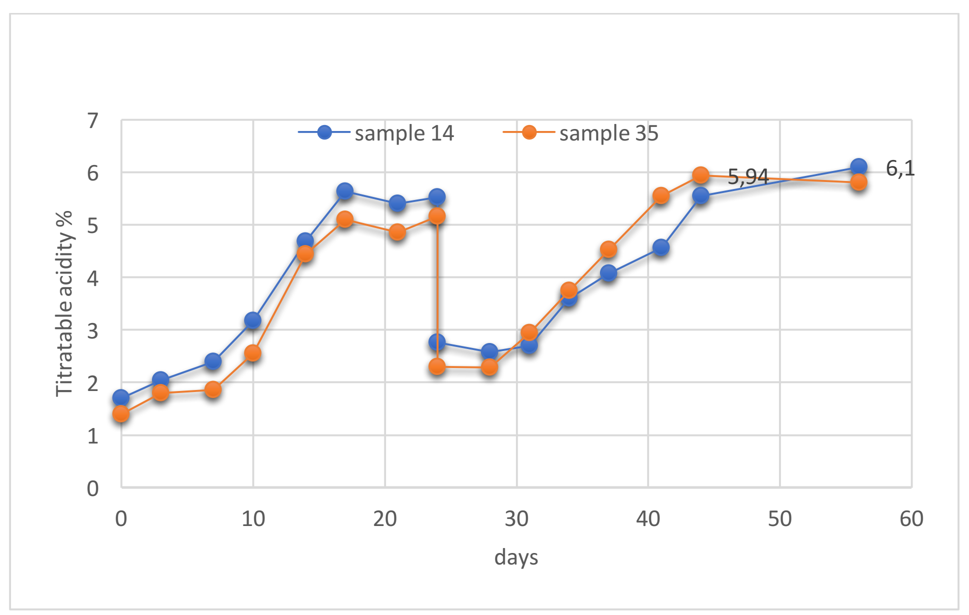

Fig. 4. Fermentation trend of fermentations conducted with strains SG14 and SG35.

Results of this study showed that is possible to apply a surface culture fermentation **from company's strain cultures** recovered from fermenting vinegars, allowing to design an **innovative vinegar process** starting from a conventional raw material (rice syrup) which is usually adopted for industrial scale production.

Then, strains 14 and 35 were considering suitable to produce rice vinegar which acetic acid content range from 5.8 to 6.1.

5.4 References

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General Conclusions

General Conclusions

Starting from the experience of the company Acetaia San Giacomo, with over 20 years of activity in premium and luxury segment of vinegar market, the conceptual analysis adopted in this study was driven by a segmented market approach.

First an analysis of vinegar market and regulatory context was assessed as essential step to focus the research.

Vinegar consumption is growing globally but the sector must face different changes.

The first is related to food consumption in general, with a constant increasing of "Out-of-Home" sector. Therefore, the research had also in mind restaurateurs and hospitality management's needs.

The second is due to the perceived value of this staple condiment, basic and therefore key ingredient in almost any kitchen of the world. Industry set its productive approach focusing of high quantity with less regards to quality.

These productive and normative choices, reflects on retail price. And vice versa.

Low sell-out price reflects also on the application of researches performed so far, to industrial manufacturing environment.

But nowadays there are new opportunity emerging from the market. We assessed a strong trend of shifting from "Traditional" vinegars to "new" kind of vinegar able to responds to new upcoming needs and values.

Emblematic is the trend observed for "Balsamic Vinegar of Modena PGI". This industrial product, taking advantage of PGI traditional EU schemes, could be considered one of the best success in the last 30 years for agri-food business. Became rapidly the fourth biggest Geographical Indication product of Europe with 92% of the production, exported.

But in the last years the trend is slowing down and decreasing, suffering of typical issue of "mature-market" products (for instance, effect on sells of the promotional prices).

The current shifting in consumer's desire is proven by the reaction of the biggest PGI Balsamic Vinegar of Modena manufacturers (that, not occasionally, were also among the biggest producers of common wine vinegar, which has been taken over by the "Balsamic Vinegar").

They are in fact pivoting their product and production line, extending to new product nowadays based on apple, making vinegar with healthier claim and different flavor profile and also adopted as base for drink; this follow the growing demand for fermented sour drink started from other

countries that are perfectly receipted by the new generations of consumer (eg. Kombucha tea, Kefir, drinking vinegars typical of eastern countries).

In addition to other generalist tendencies such as sustainability and health awareness, "fermentation" seems to be a transversal driver capable of:

1) intercepting new trends (healthy and different flavor profile)

2) if performed in a particular way, is able to enable a high market positioning, thanks to added values, different from the functional and utilitarian ones, and more linked to a hedonistic domain (slow, natural, limited edition, not standardized, done with care, made with higher labor/tech ratio).

Also self-production of fermented foods show a growing trend, either in domestic or restaurant business, able to respond to “self-fulfillment” needs (and more sustainability and healthiness).

In the present research, we defined the claim that a static fermentation can legitimately adopt, as "artisanal".

This industrial PhD thesis performed a horizontal investigation starting from the aforementioned assessing. Look for new space for incremental innovation in vinegar market and investigate how and where the static acetic fermentation (as implicit feature able to trigger hedonistic added value), can be performed.

From this outlook both new products and new processes were investigated with regards of:

1) PROCESS: resize and adapt the static acetic fermentation to a "domestic" scale for not professional users, allowing a self-production (also intended in a circular economy approach) in an affordable and user-friendly way.

2) PRODUCTS:
    a. Unconventional raw materials, leftover from cheese production with pollution issues related: cheese whey as fermentative substrate for an innovative non-alcoholic healthy beverage or low-acidity condiment suitable for food preparation (marinating).
    b. Unconventional raw material for western vinegar’s market, with great potential with regards of availability and customer’s acceptance: rice (saccharified rice syrup) as fermentative substrate for low acidity condiments or vinegars production.

In the processes and products analyzed, the static fermentation has been successful used as a biochemical common denominator able to convey a set of added values, connected to the hedonistic sphere of needs.

The laboratory trials, as well as test at prototypal scale at company's facility, conducted on new raw materials (rice syrup and cheese whey) demonstrate the validity of the static superficial method to perform biochemical transformations in line with new consumer's needs.

Acetaia San Giacomo, as promoter of this industrial PhD project, will add a new product called "Italian Artisanal Rice vinegar" starting from 2020.

More investigation on cheese whey is needed in order to understand which is the best fermentative culture to adopt. However, cheese whey as raw material will be adopted to meet Company's customers (food service sector) needs. In fact thery are willing to get a low acidic seasoning for food preparation that can be appreciated for the sustainability approach.

The process innovation related to the designing and adapting the kit for domestic vinegar production by not specialized users, has already provided interest from media, private individual and restaurateurs.

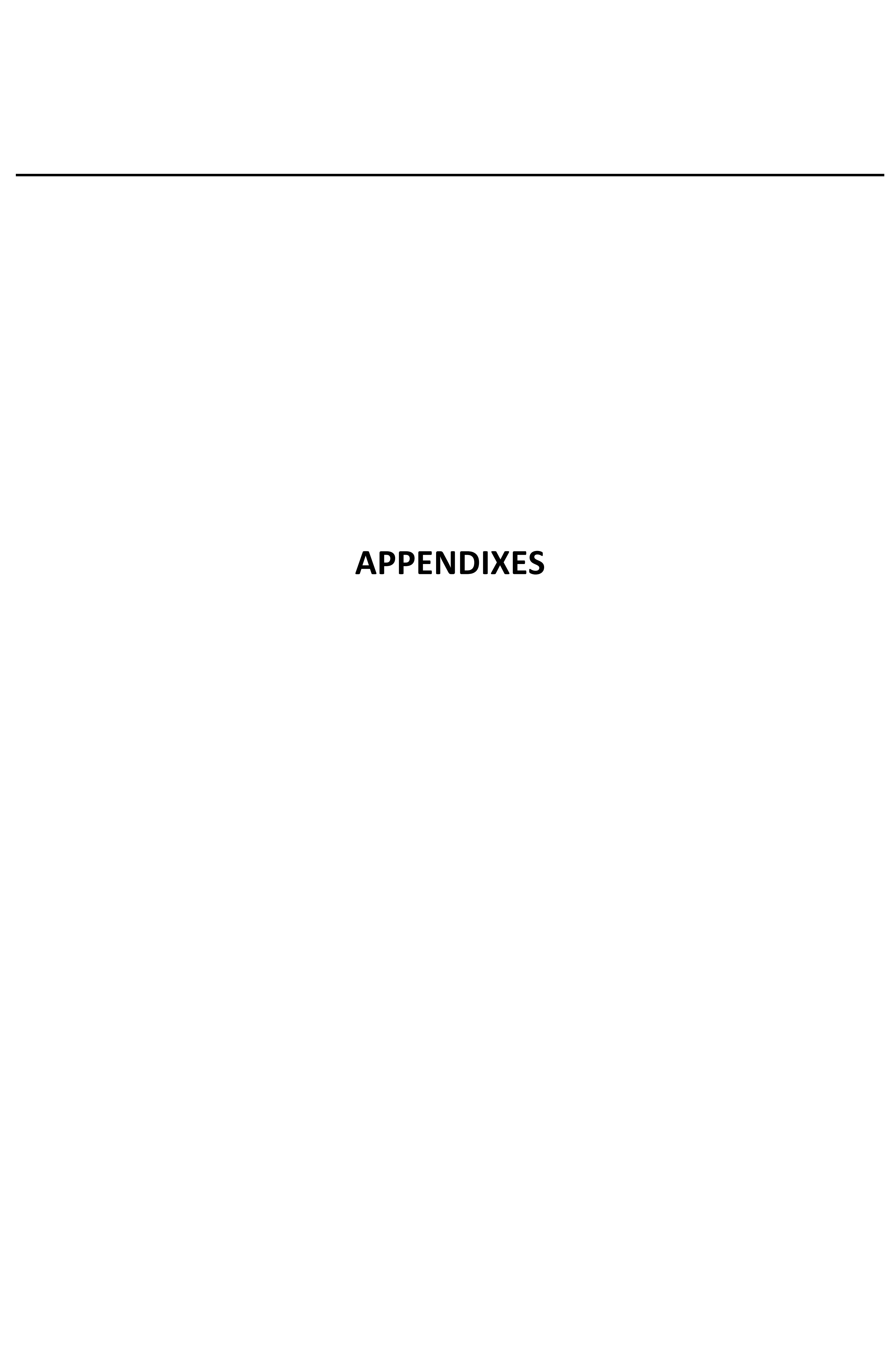
In the appendixes is possible to find some resources about the media coverage, test performed with an important high-end Italian restaurant and exhibition were the prototypal kit has been hosted.

The company also started self-production of Artisanal Balsamic Vinegar in different restaurant in United States (pictures in appendix).

RINGRAZIAMENTI

Un sentito ringraziamento va alla mia famiglia che mi ha permesso di intraprendere questo affascinante percorso e in particolare a mio padre. So che sarebbe molto orgoglioso di questo lavoro e di quanto fatto finora con le sue botticelle. Sei la mia luce quando sono perso nel buio.

Desidero inoltre ringraziare di cuore i colleghi dell’Acetaia San Giacomo che mi hanno permesso di lasciare tranquillamente l'azienda durante il tempo trascorso in questo lavoro. Non avremmo mai raggiunto vent'anni di attività commerciale e così tanti incredibili risultati senza di loro



APPENDIXES

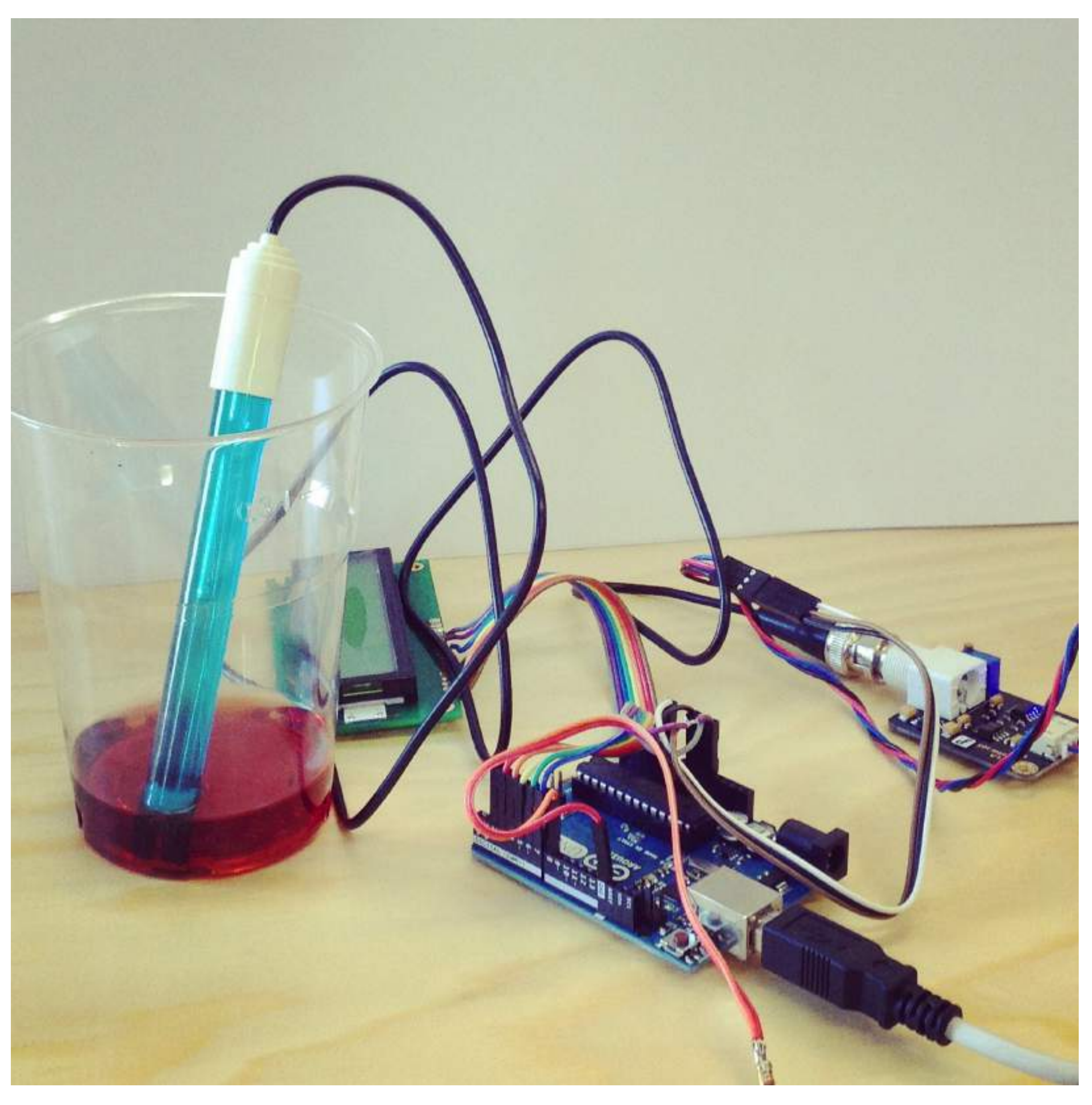
Appendix 1.
Kit for domestic production of vinegar

Idea Challenge at Reggio Emilia Fab Lab – january 2015

IDEA CHALLENGE

Acetaia San Giacomo

Gennaio 2015



13

OGGETTO: Acetificatore domestico

A partire dall'idea dell'Acetaia San Giacomo e dal relativo prototipo di acetificatore domestico, realizzato al Fab Lab con Andrea Zanzanelli e Maria Gullo (per lo sviluppo del processo di acetificazione controllato) , si richiede al gruppo di lavoro di disegnare alcune possibili soluzioni di design per integrare le varie componenti e rendere l'oggetto più funzionale. E' possibile pensare a strategie di brand, modalità di diffusione e potenziali target.

Location: Fab Lab Reggio Emilia @Musei Civici - Reggio Emilia

Programma:

ore 9.00 - Introduzione- obiettivi del challenge
ore 9,15 - Presentazione Andrea Bezzecchi Acetaia San Giacomo
ore 9,30 - Maria Gullo – Unimore Microbiologia degli Alimenti -UMCC-

- *Il mistero della trasformazione del vino*
- ore 10,00 - Presentazione del gruppo e impressioni a caldo

ore 10,30 - Coffee break
ore 12.00 - Primo confronto sui concept – Coordinamento tutors
ore 13.00 - Lunch Break ?
ore 14.00 - Gruppi di lavoro
ore 15.00 - Secondo confronto sulla fattibilità – Tutors –Fab Lab
ore 16.00 - Prototipazione
ore 18.00 - Presentazione dei progetti
ore 18.30 - Aperitivo e saluti e standing ovation

Attori coinvolti:

- **Andrea Bezzecchi** Acetaia San Giacomo
- **DQUID** - Vito Poleo
- **Giulia Morselli** – Designer/illustratrice giuliamorselli@libero.it
- **Andrea Ferrari –** Maker info@afproject.it
- **Giuseppe Tortora –** Tecnico elettronico tortora.electronics@yahoo.it
- **Claudio Storchi –** Product design
- **Piergiorgio Grossi –** Manager –Better Decision-
- **Antonio Starnino –** FIP – Product manager
- **Maria Gullo** Unimore - Laboratorio Microbiologia degli Alimenti -UMCC
- **Gabriele Zanichelli –** Dottorando di ricerca - Unimore - Laboratorio Microbiologia degli Alimenti

Tutors :
- **Fernando Arias** – Prototipazione rapida e fabbricazione digitale ferna.arias77@gmail.com
- **Andrea Zanzanelli –** Ingegnere meccatronico, maker andrea.zanzanelli@gmail.com
- **Claudia Parisoli –** Esperta in scienze della comunicazione

Coordinamento e controllo qualità:

- **Francesco Bombardi-** Architetto , Responsabile Fab Lab Reggio Emilia, Professore a contratto Dipartimento di Scienza e Metodi dell' ingegneria UNIMORE. bombardiarchitetto@gmail.com

Guest: Stefano Maffei stefano.maffei@polimi.it

Fab Lab Reggio Emilia è un laboratorio di fabbricazione digitale di ultima generazione, nato in seno a Reggio Emilia Innovazione e segnalato dal Ministero dell'Istruzione come "best practice" nella Social Innovation Agenda 2013. Il laboratorio svolge attività di formazione e ricerca all'interno dei Musei Civici, gestendo un'importante rete di risorse umane (creativi/talenti) a seconda delle richieste/esigenze delle imprese e secondo processi di partecipazione/produzione innovativi.

Alcune attività del Fab Lab Reggio Emilia si possono vedere su www.fablabreggioemilia.org

Laboratorio microbiologia degli alimenti. Dipartimento di Scienze della Vita, Università degli Studi di Modena e Reggio Emilia
Svolge attività di ricerca nel campo della microbiologia degli alimenti fermentati e dei processi correlati. Attraverso la creazione e la gestione della collezione microbica (UMCC) offre servizi per la ricerca, l'insegnamento e l'industria biotecnologica. Le attività di UMCC comprendono in particolare la selezione di microrganismi per specifiche applicazioni biotecnologiche, lo sviluppo di processi di fermentazione e relativo trasferimento tecnologico.

Acetaia San Giacomo è un luogo di prosecuzione della Tradizione famigliare nella produzione di Aceto Balsamico Tradizionale di Reggio Emilia Dop ed altri prodotti fortemente legati alla territorio.
Medaglia d'oro dalla Camera di Commercio come "Impresa Giovane" (età media degli addetti, misure innovative adottate) e "Sede didattica" dell'Università di Scienze Gastronomiche di Pollenzo, è meta degli amanti del gusto che, da ogni parte del mondo, giungono per una visita e per assaporare l'atmosfera dei luoghi dove il tempo sembra essersi fermato.

Fab Lab Reggio Emilia propone alle aziende varie formule di collaborazione tra cui l'IDEA CHALLENGE, che si sta rivelando particolarmente efficace. Si tratta di una attività, regolata da contratto di ricerca, che prevede:

1) preparazione insieme all'azienda di un brief dettagliato su un tema / prodotto da sviluppare

2) organizzazione di 1-2 giornate di lavoro negli spazi del Fab Lab che prevederanno la presentazione del brief e lo sviluppo di concept/soluzioni attraverso gruppi di lavoro interdisciplinari con tutoraggio e facilitazione da parte di esperti con l'obiettivo di arrivare alla prototipazione delle migliori idee. Alla fine delle giornate di lavoro si può prevedere la presentazione pubblica delle proposte attraverso la pianificazione e comunicazione di un evento.

3) Sintesi e finalizzazione delle proposte da consegnare all'azienda

4) Eventuale approfondimento e sviluppo esecutivo di una o più proposte

La formula dell'Idea-Challenge costituisce un'opportunità per le aziende di promuovere la ricerca su temi di interesse attraverso azioni mirate e che abbiamo una immediata risposta nella produzione materiale di prototipi. Attraverso la contaminazione e la visione "laterale" generata da gruppi di lavoro eterogenei ed interdisciplinari è possibile spostare i punti di vista e provocare gli stimoli e le azioni che possono innescare processi di innovazione.

I gruppi di lavoro coinvolti saranno scelti sulla base delle qualità e competenze già rivelate spontaneamente nelle attività programmate durante l'anno dal Fab Lab e avranno la possibilità di apprendere i processi della fabbricazione digitale e di conoscere da vicino realtà d'impresa innovative e persone con cui eventualmente costruire opportunità di collaborazione successive. L'azienda si impegna a citare il Fab Lab e gli ideatori delle soluzioni che eventualmente potranno essere sviluppate e di cui i partecipanti a titolo gratuito cedono i diritti economici di sfruttamento.
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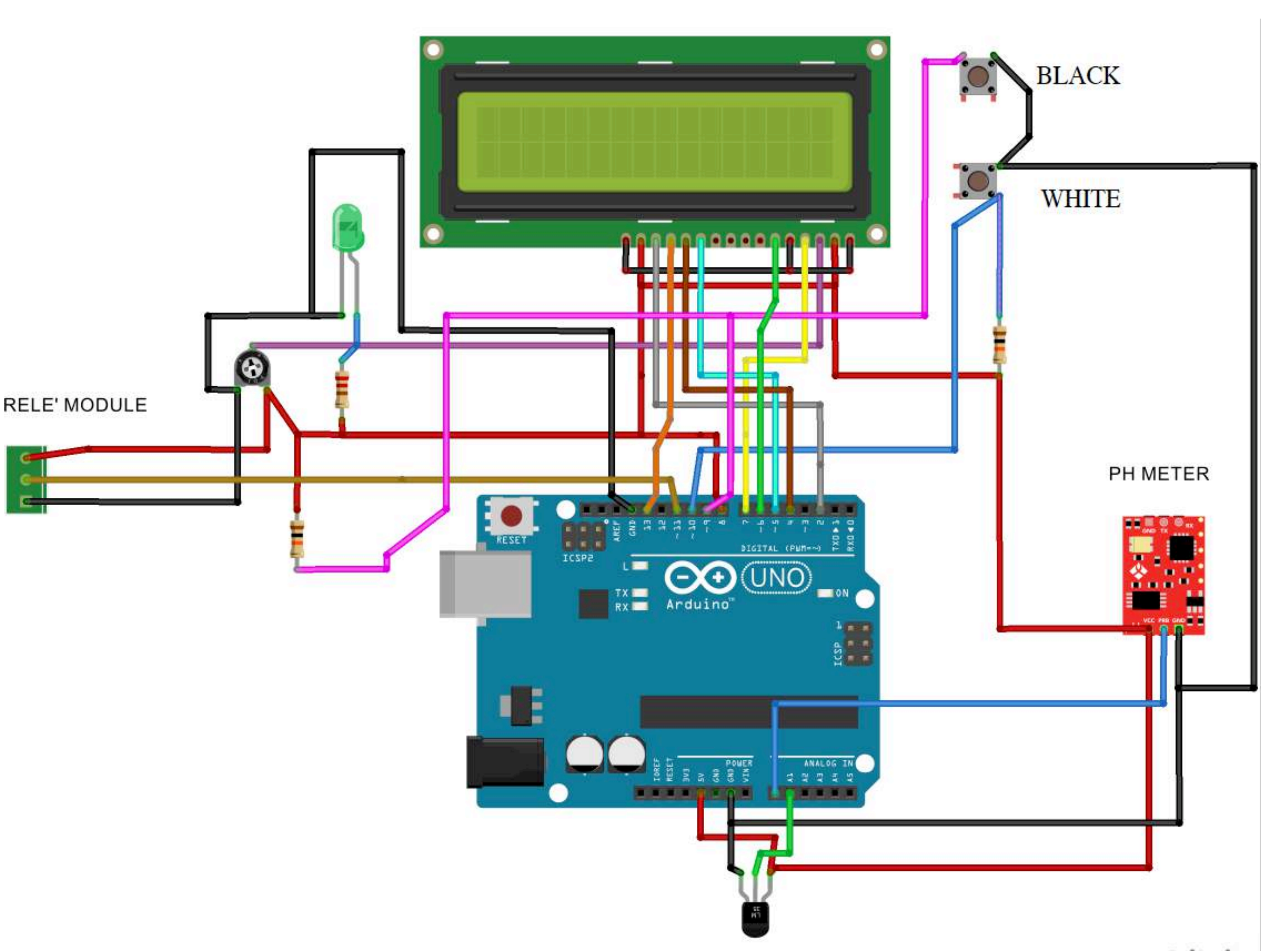
Idea Challenge at Reggio Emilia Fab Lab – january 2015
First circuit design for pHMeter and temperature control



Exhibition at Milan XXII Triennale – New Craft installation

IN OCCASIONE DELLA **XXI TRIENNALE DI MILANO**, NELL'AMBITO DELLA MOSTRA NEW CRAFT CURATA DA **STEFANO MICELLI** E OSPITATA NELL'AREA KITCHEN TOOLS PRESSO EX FABBRICA DEL VAPORE, **FRANCESCO BOMBARDI** PROPONE IL CONCEPT DI UN ORIGINALE SCENARIO DOMESTICO DOVE I NUOVI ORIZZONTI DELLA CULTURA DELL'AUTOPRODUZIONE INCONTRANO LE OPPORTUNITÀ CRESCENTI GENERATE DALLA DIFFUSIONE DELLA FABBRICAZIONE DIGITALE.

GRAZIE AL SUPPORTO DI AZIENDE COME **BARILLA**, LEADER MONDIALE NELLA PRODUZIONE DI PASTA E DA SEMPRE DEDICATA ALL'INNOVAZIONE E ALL'ESPLORAZIONE DELLE TECNOLOGIE EMERGENTI, E **MARRONE**, DA PIÙ DI QUARANT'ANNI NEL SETTORE DELLE CUCINE MONOBLOCCO SU MISURA AL SERVIZIO DELLE ENERGIE CREATIVE DI CHEF DI TUTTO IL MONDO,

QUESTA VISIONE PRENDE CORPO IN UN ALLESTIMENTO CHE PERMETTE DI VIVERE LA RICCA ESPERIENZA DELLA TRASFORMAZIONE DEL GRANO, DAL CHICCO NEL MULINO, ALLA FARINA FINO AD ARRIVARE A STAMPARE FORME DI PASTA IN 3D.

SULLA SCIA DELL'ESPERIENZA INTERNAZIONALE DEL FOOD INNOVATION PROGRAM E DELL'ECOSISTEMA DI VALORI DIFFUSI SUL TERRITORIO LOCALE SI GENERA L'INCONTRO DI REALTÀ COME L'AZIENDA **ACETAIA SAN GIACOMO**: CHE PRESENTERÀ FACETO, IN ANTEPRIMA PER KITCHEN TOOLS, LA MACCHINA PER FARE L'ACETO IN CASA ATTRAVERSO UN SISTEMA DI ACCELERAZIONE E CONTROLLO TRAMITE SENSORI.

HESTIA DESIGN LAB DI HILDRETH ENGLAND, CHE PERMETTE DI SPINGERE IL PROGETTO VERSO LIMITI ANCORA PIÙ AMBIZIOSI, ESPLORANDO L'UNIVERSO DELL'INTERACTION E BEHAVIOUR DESIGN IN COLLABORAZIONE CON **DQUID**, AZIENDA EMERGENTE NEL MONDO DELL'INTERNET OF THINGS.

SI AGGIUNGONO POI 2 PROGETTI DEGLI STUDENTI UNIMORE, ENRICO FRANCHINI, ENRICA BONILAURI, TOMMASO FRATTINI PER **DISPENSA 2.0** E MICHAEL FILI E ALFREDO ADINOLFI BOREA PER **@ROMATIC**.

LO SVILUPPO DEI PROTOTIPI E LA CONSULENZA TECNICA PER L'INTERAZIONE È COORDINATA DA **WONDERLAB** DI **LUCA CROTTI**.

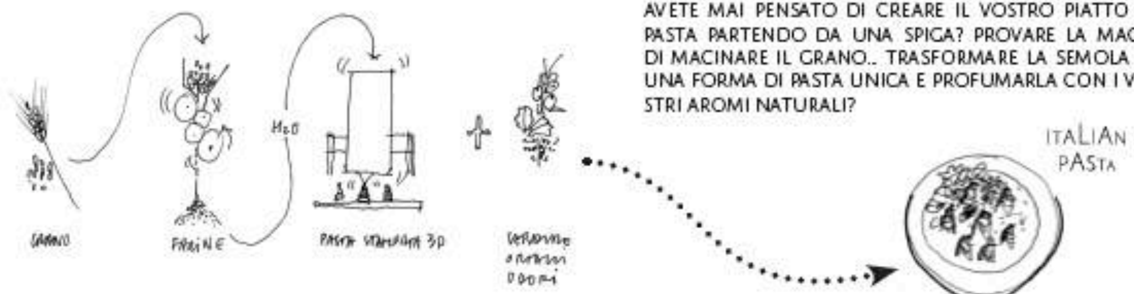
PAOLO TEGONI, DISEGNA LA PIATTAFORMA WEB E L'ARCHITETTURA DELLA APP CHE CONSENTE AL VISITATORE DI SIMULARE UNA ESPERIENZA DOMESTICA DI INTERAZIONE CON LA CUCINA, ATTRAVERSO AZIONI GUIDATE E COMPATIBILI CON DIETE PERSONALIZZATE.

LA VIDEO INSTALLAZIONE CHE ACCOMPAGNERÀ I VISTATORI PER TUTTO IL PERIODO È REALIZZATA DA CLAUDIO PICCININI, ALESSIO FERRERA E FEDERICO NOCCO DI **RED CREATIVE COMPANY**.

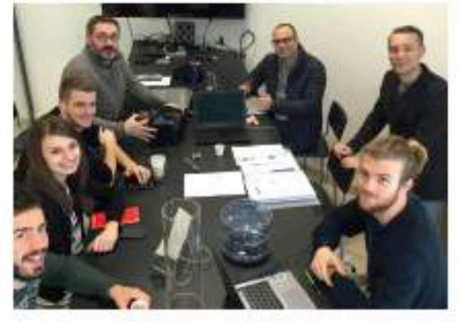
GIANMARIA SFORZA PROPONE UNA PERSONALE SELEZIONE DI STRUMENTI PER IL NUOVO SCENARIO DOMESTICO.

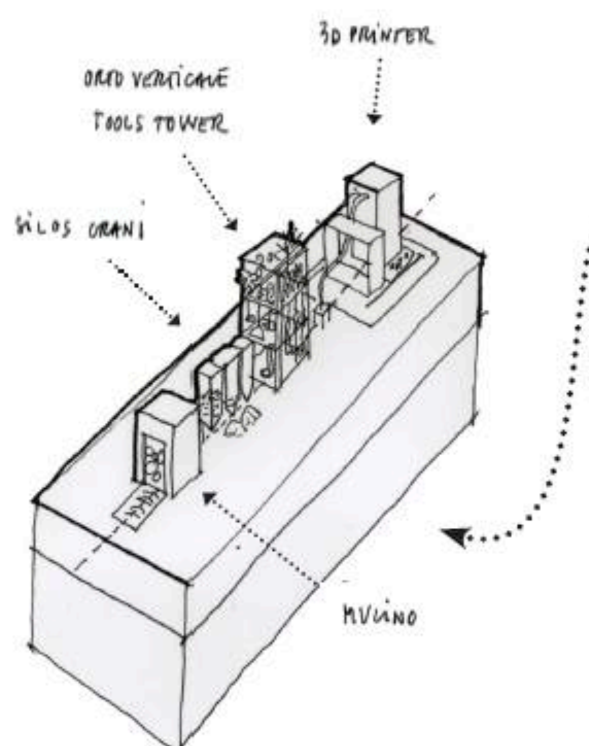
13

AVETE MAI PENSATO DI CREARE IL VOSTRO PIATTO DI PASTA PARTENDO DA UNA SPIGA? PROVARE LA MAGIA DI MACINARE IL GRANO.. TRASFORMARE LA SEMOLA IN UNA FORMA DI PASTA UNICA E PROFUMARLA CON I VOSTRI AROMI NATURALI?



LA XXI TRIENNALE DI MILANO METTE IN SCENA QUESTA ESPERIENZA, CHE PRESTO POTREMMO IMMAGINARE TRASPORTATA IN AMBITO DOMESTICO. SE POI AGGIUNGIAMO UN TEAM DI RICERCA COSTITUITO DA PROFESSIONISTI ESPERTI IN BEHAVIOUR DESIGN E INTERACTION DESIGN È POSSIBILE ANCHE PENSARE AD UNA ESPERIENZA ASSOLUTAMENTE ORIGINALE DI INTERAZIONE, CON FORMULE PERSONALIZZATE A SECONDA DEL PROPRIO FABBISOGNO NUTRIZIONALE.





IL GRANO È RACCOLTO IN PICCOLI SILOS, PRONTO PER ESSERE MACINATO ALL'INTERNO DEL MULINO E TRASFORMATO IN SEMOLA FRESCHISSIMA, CHE VERRÀ CONSERVATA IN ALTRI SILOS ADIACENTI.

TRAMITE L'APPLICAZIONE PREDISPOSTA, L'UTENTE PUÒ DECIDERE LA QUANTITA' E LA TIPOLOGIA DI SEMOLA IN BASE AL PROPRIO STILE DI VITA.

TALE MIX È COMMISURATO AL PROPRIO FABBISOGNO ENERGETICO: CONSIDERA LE CALORIE CONSUMATE NELLA GIORNATA E I PARAMETRI PERSONALI.

KITCHEN TOOLS SI ISPIRA A **AURASANO**, CONCEPT SVILUPPATO DA HESTIA DESIGN LAB CHE GIOCA CON LUCI E SUONI PER GUIDARCI DELICATAMENTE ATTRAVERSO I PROCESSI DI PREPARAZIONE DEI PASTI, PERSONALIZZATI SULLE NOSTRE PREFERENZE E IL NOSTRO STATO DI SALUTE. AURASANO INCARNA LA VISIONE DI UNA CUCINA CHE CI COINVOLGE IN UNA CONVERSAZIONE SU CIBO, SALUTE E BENESSERE IN MODI PIACEVOLI E SORPRENDENTI.

PER OTTENERE IL PIATTO IDEALE IL PASSO È BREVE: È SUFFICIENTE INSERIRE L'IMPASTO NELLA STAMPANTE 3D, SELEZIONARE IL TIPO DI PASTA CHE SI DESIDERA E IN POCHI MINUTI SI OTTERRÀ LA PROPRIA PASTA PERSONALIZZATA, PRONTA PER LA COTTURA DA ARRICCHIRE CON GLI ODORI COLTIVATI ORTO IDROPONICO

EVENTI

A CURA DI: BARILLA, FUTURE FOOD INSITUTE, FOOD INNOVATION PROGRAM, SIMONE ARDIGÒ, CESARE PUZZI, EMILIO ANTINORI CON ABACO SOC.COOP, SLOWD, TOURDEFORK, OPEN DOT, ANDREA PATERNOSTER, ANDREA BEZZECCHI

FUTURE FOOD INSTITUTE
FOOD INNOVATION PROGRAM

2 APRILE	OPENING
28 APRILE	BEZZECCHI -FACETO
3 MAGGIO	SLOW/d - VINEGRAAL
17 MAGGIO	BARILLA
18 MAGGIO	EMILIO ANTINORI
26 MAGGIO	TOURDEFORK - FOOD MACHINES
31 MAGGIO	ARDIGO' - FAUNA MACROBENTONICA + PATERNOSTER - HONEY DOMESTIC
17 GIUGNO	UNIMORE CORSO FOOD DESIGN WORKSHOP .FBOMBARDI
24 GIUGNO	UNIMORE CORSO FOOD DESIGN WORKSHOP .FBOMBARDI
6 LUGLIO	BARILLA
7 SETTEMBRE	BARILLA

DATE DA CONFERMARE

GLI STUDENTI DEL CORSO DI INGEGNERIA GESTIONALE - PROF. MATTEO VIGNOLI - SCELGONO COME TEMA PER IDEA CHALLENGE LA VALORIZZAZIONE DEL LAVORO DI TEAM

Exhibition at Milan XXII Triennale – New Craft installation
Loan Form

Fondazione
La Triennale di Milano
viale Alemagna 6
20121 Milano

tel. 39 2 724341
fax 39 2 89 01 0693
telex 311272 Trienn I
codice fiscale/fiscal code
01423890159

Foglio di prestito
Loan form

Mostra/exhibition **NEW CRAFT**

Periodo di svolgimento/opening period **2 aprile – 12 settembre 2016**

Sede espositiva /exhibition venue: **FABBRICA DEL VAPORE (via Procaccini 4, Milano)**

Tipologia dell'opera/oggetto
Type of work
KIT PER LA PRODUZIONE DOMESTICA DI ACETO

Artista/Autore
Name of artist/author
ANDREA BEZZECCHI - ACETAIA SAN GIACOMO

Titolo dell'opera
Title of work/item
FACETO

Anno di realizzazione
Date of execution
2015

Materiale, tecnica, peso in Kg
Material, technique, weight
AMPOLLA IN VETRO SOFFIATO, AREATORE,
SCHEDA ELETTRONICA, PHMETRO DIGITALE
4 KG

Numero d'inventario/serie
Inventory or serial number

Dimensioni di ogni opera in cm Dims of each work/item	Altezza Height	Larghezza Width	Profondità Depth
45 X45 X40 H	40	45	45

Valore assicurativo
Insurance value
350,00 EU

Eventuali particolari condizioni per trasporto e assicurazione
Special conditions for transport and insurance
IMBALLARE CON CURA L'AMPOLLA IN VETRO SOFFIATO
E LE PARTI ELETTRONICHE

Nome e indirizzo del prestatore
Name and address of the lender
ANDREA BEZZECCHI
STRADA PENNELLA, 1
42017 NOVELLARA (RE)

Telefono e fax
Telephone and fax number
0522651197 - 3487841122

Come desidera essere menzionato?
How does the lender wish to be mentioned?
IDEATORE

1 di/of 2

14

Indirizzo presso cui ritirare l'opera/oggetto
Address at which the work is to be collected

Indirizzo presso cui restituire l'opera/oggetto
Address to which the work is to be returned

ACETAIA SAN GIACOMO

STRADA PENNELLA, 1

42017 NOVELLARA

Autorizza la riproduzione dell'opera/oggetto per il catalogo, i materiali di comunicazione, la stampa e la televisione?
May the work/item be reproduced in the catalogue, the communication materials, the press and on television?

SI

Autorizza il prolungamento del prestito dell'opera/oggetto in caso di proroga dell'apertura al pubblico o di itineranza della mostra?
May the loan of the work/item be extended if the exhibition is prorogated or to other possible venues of the exhibition?

SI

Note
Notes

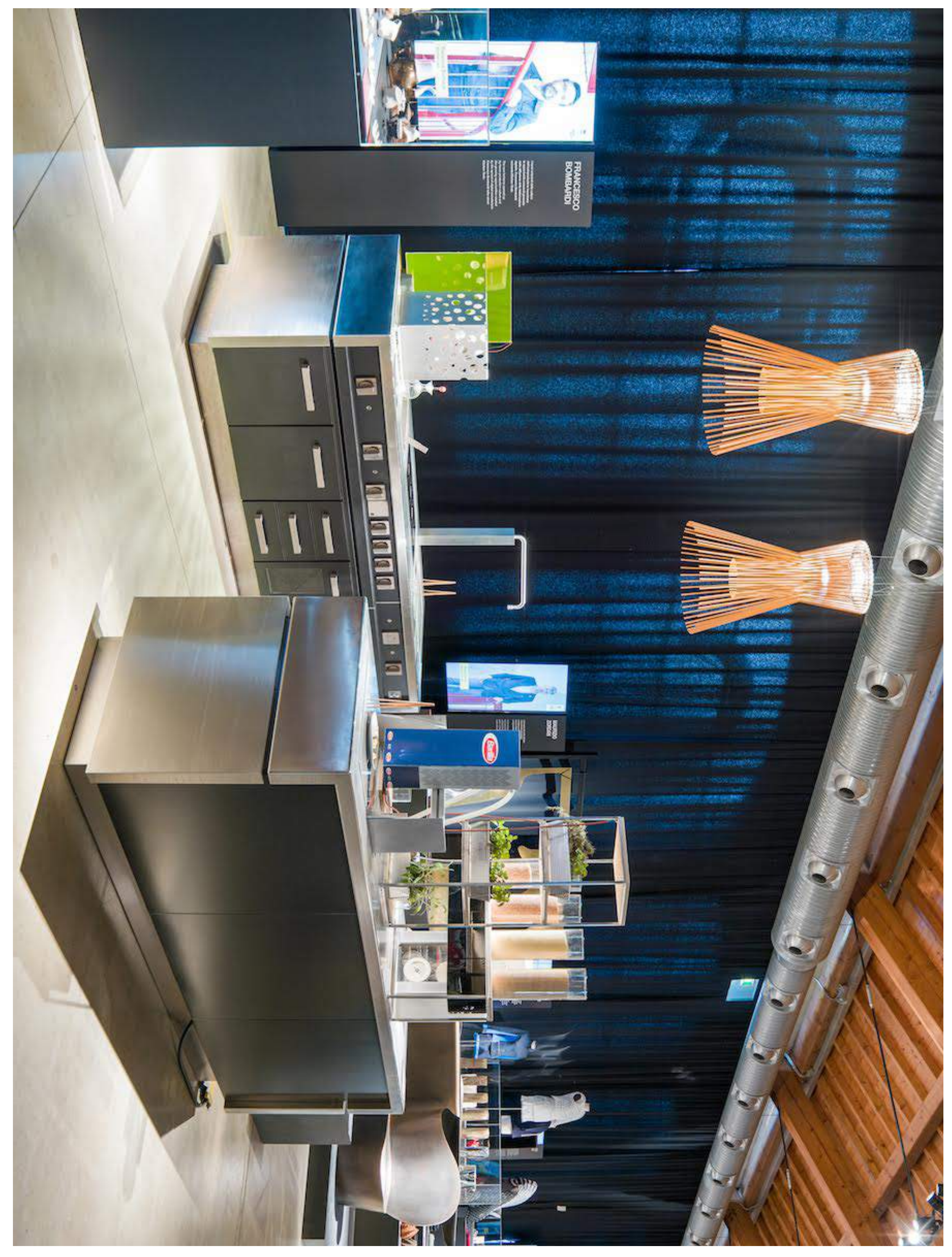
Data
Date 16/5 /2016

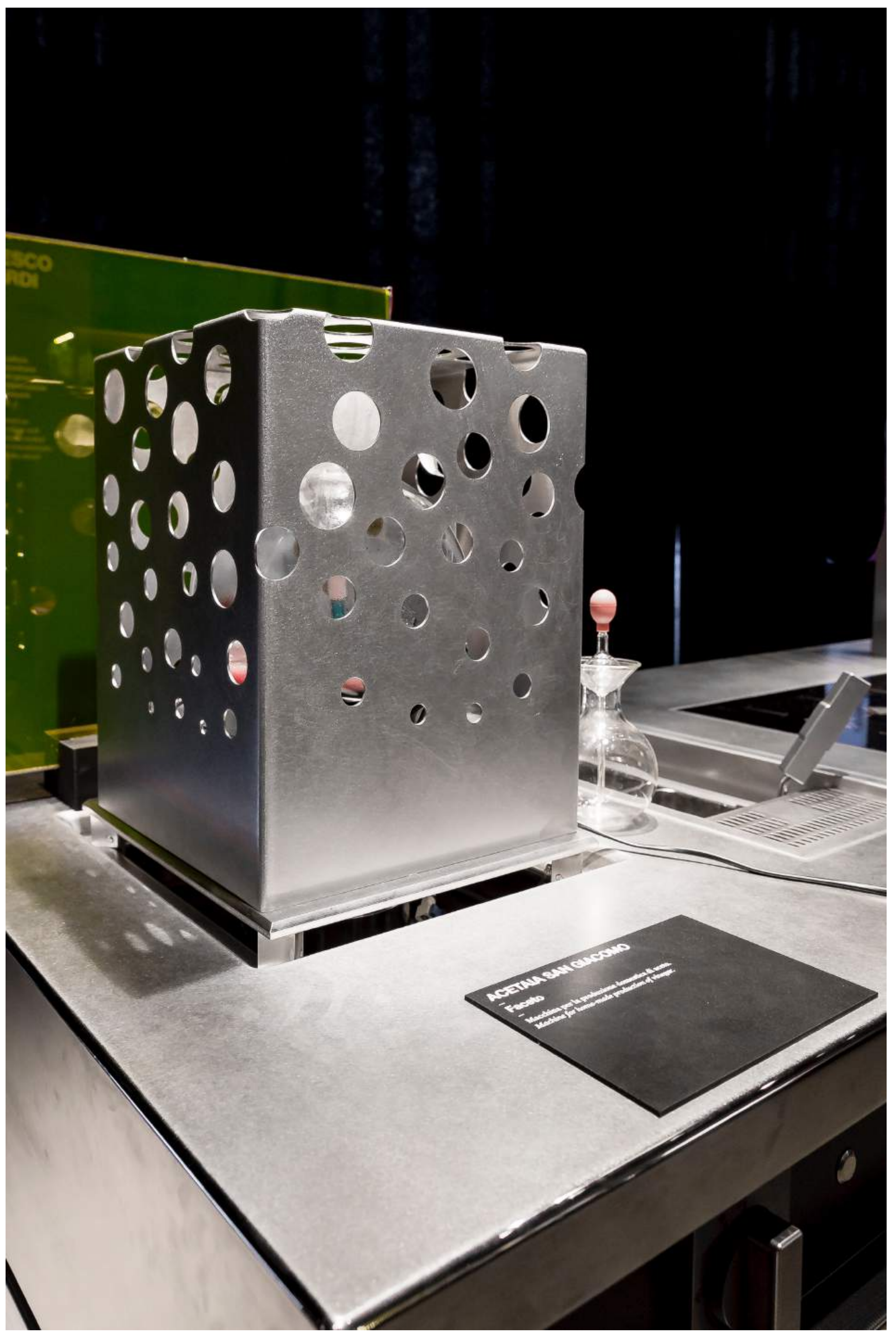
Fondazione La Triennale di Milano
Il Direttore Generale/The Director General
Andrea Cancellato

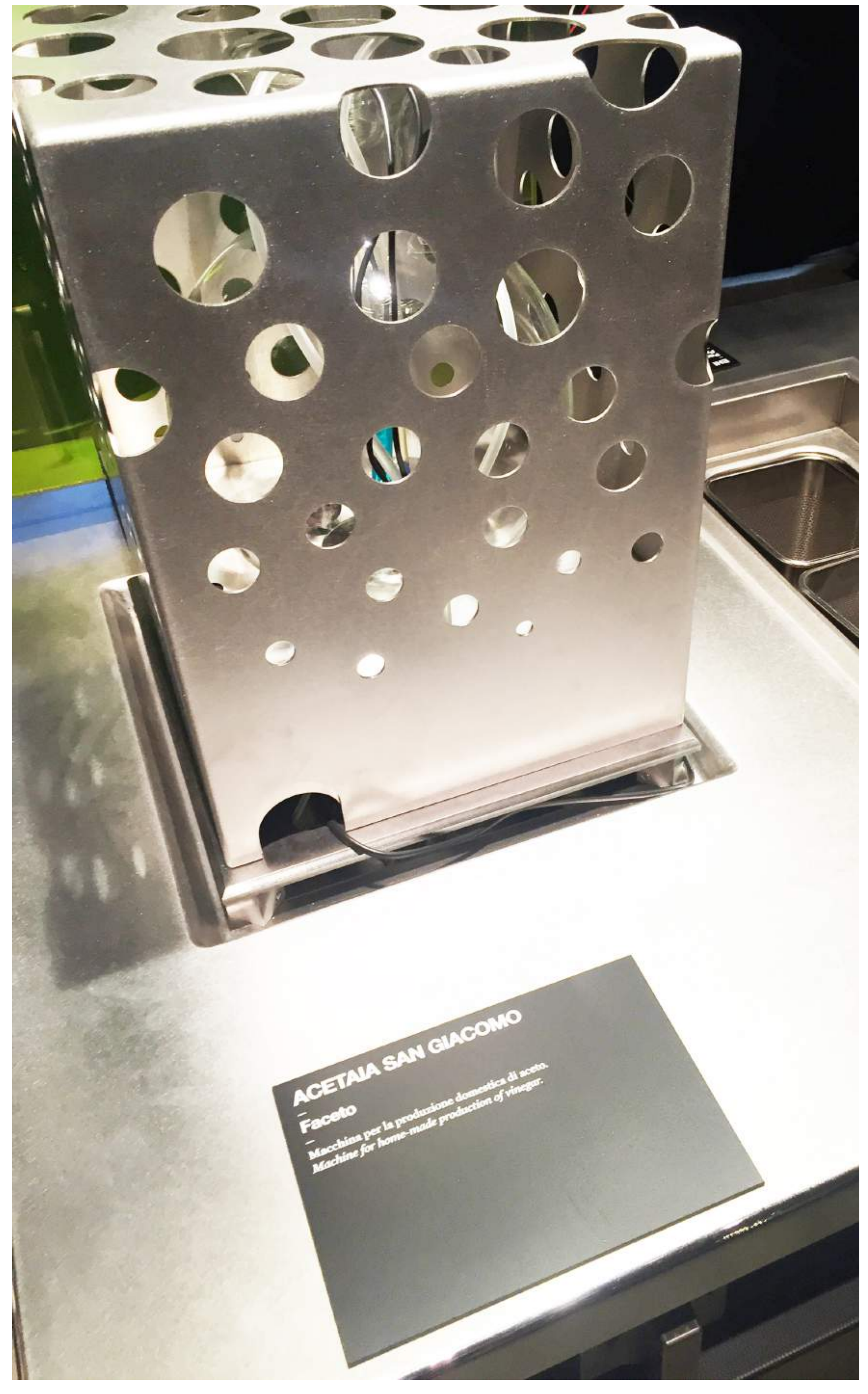
Il Prestatore
The Lender

Appendix 5.
Kit for domestic production of vinegar

Exhibition at Milan XXII Triennale – New Craft installation
Pictures




ACETAIA SAN GIACOMO
Faceto



14

Media coverage after the Maker Fair Rome exhibition
Rai Radio 1 – Eta Beta January 4th 2016
Corriere della Sera online

Rai Play Radio

quando la tecnologia reinventa un antico mestiere

Vai al programma Aggiungi a Playlist Condividi

Cosa nasce se linventiva degli artigiani italiani si sposa con le straordinarie possibilità dellinnovazione digitale? È la domanda su cui riflette la nuova puntata di Eta Beta: il reciproco beneficio che può generare lincontro tra un sapere antico e una conoscenza moderna.

Cosa nasce se l'inventiva degli artigiani italiani si sposa con le straordinarie possibilità dell'innovazione digitale? È la domanda su cui riflette la nuova puntata di Eta Beta: il reciproco beneficio che può generare l'incontro tra un sapere antico e una conoscenza moderna.

Ospiti: **Stefano Micelli**, docente di Economia e Gestione delle imprese presso l'Università Ca' Foscari di Venezia e autore del Best seller "Futuro artigiano";
Andrea Bezzecchi, ideatore di Faceto, kit tecnologico per prodursi l'aceto balsamico dentro casa;
Leonidas Paterakis, designer di mobili le cui forme possono essere decise al computer dai committenti;
Matteo Zugnoni, fondatore di Woobi, la bicicletta in legno;
Paola Volpi, disegnatrice di gioielli grazie alla stampante 3D;
Sara Savian, coidestrice di Digital Fashion.

Ascolta l'audio

Rai Radio 1
Eta Beta del 04/01/2016 - Artigiani, quando la tecnologia reinventa un antico mestiere
Eta Beta

CORRIERE DELLA SERA

Maker Faire tra stampa 3D, robot, orti portatili e skateboard in aghi di pino

3 / 66 Slide Show

Faceto realizza l'aceto in casa

Raw test for ethanol sensor – Ing. Luca Crotti

TEST DI VALUTAZIONE dell'EFFICACIA di LETTURA di SENSORI PER LA RILEVAZIONE DI ALCOL ETILICO UTILIZZATI PER LA VALUTAZIONE DELLO STADIO DI ACETIFICAZIONE DI VINO

1. SCOPO

L'obiettivo del test è verificare le letture di alcuni sensori (nome commerciale MQ-3) in grado di rilevare la concentrazione di alcol etilico, utilizzati però per verificare l'evoluzione di vino in fase di acetificazione.

Lo scopo finale dell'analisi è quella di poter utilizzare detti sensori in un prodotto in fase di sviluppo in grado di controllare il processo di acetificazione casalingo

2. DEFINIZIONI e SPECIFICHE

TECNICHE SENSORE UTILIZZATO:

I sensori di alcool maggiormente diffusi attualmente sono l'MQ-3 (immagine a fianco) prodotto in Cina dalla Hanwei Electronics, e il TGS822 prodotto dalla Giapponese Figaro.
Tali sensori sono costituiti all'interno da un tubicino in materiale ceramico (Al2O3) intorno al quale è depositato un film sottile di Biossido di Stagno (SnO2).

Questi sensori hanno una sensibilità molto elevata: possiamo difatti notare come in presenza di piccole quantità di gas nell'ambiente, la tensione in uscita dal partitore aumenta in maniera repentina.
Hanno però numerosi svantaggi tra cui la durata (non hanno una vita molto lunga e necessitano di essere sostituiti dopo alcuni anni di funzionamento) e la risposta non lineare. Il loro basso costo e la loro semplicità di uso però ne hanno di molto diffuso l'utilizzo.

Il Biossido di Stagno è un semiconduttore, più precisamente un semiconduttore di tipo N: ovvero tale materiale viene drogato (vengono cioè aggiunti elementi chimici alla sua struttura) in maniera tale che vi sia al suo interno un eccesso di elettroni liberi che sono i responsabili
della conduzione di corrente elettrica.

E' lo stesso sistema che si utilizza per i normali semiconduttori:
diodi, leds, transistors ecc.

Questofilmsottiledibiossidodistagnoinoltreèporoso,ilchevuol dire che presenta dei microfori all’interno dei quali può facilmente penetrare l’ossigeno ambientale. In condizioni normali l’ossigeno viene adsorbito da questo film sottile, sottraendo elettroni alla struttura secondo lareazione:

Il Biossido di Stagno ha un eccesso di elettroni; l’ossigeno entrando all’interno del semiconduttore, sottrae questi elettroni (e–) con la conseguenza di far diminuire la conducibilità del materiale (o in altre parole: ne fa aumentare la resistenzaelettrica).

In queste condizioni, quindi, la misurazione di una resistenza molto elevata sul sensore può farci capire che nell'ambiente è "normale". Nell'applicazione prototipale sviluppata, il sensore viene posto in un circuito a partitore di tensione in maniera tale da poter misurare la tensione in uscita e fare quindi le opportune conversioni:

I vapori di alcool subiranno il seguente processo:

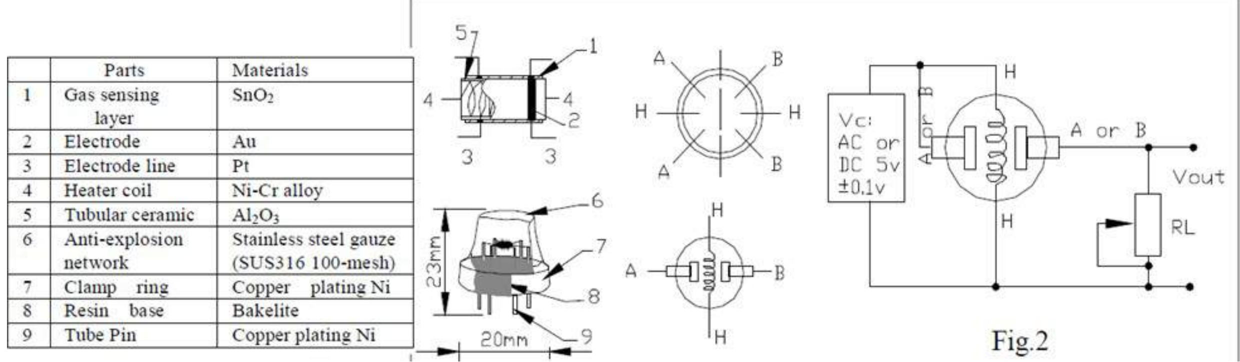
$$CH_3CH_2OH + 2O^- \rightarrow CH_3COOH + H_2O + 2e^-$$

(questa non è precisamente la reazione che avviene ma ne è soltanto il "prodotto finale" e rappresenta una semplificazione)
In pratica l'alcool (CH3CH2OH) reagisce ossidandosi ad acido acetico (CH3COOH) ed acqua (H2O) e (cosa importante) liberando elettroni.
Gli elettroni liberati aumentano la conducibilità del materiale, di conseguenza la resistenza elettrica diminuisce causando un aumento di Vout nel partitore di tensione.
L'alcool in effetti non si ossida direttamente ad acido acetico ma passa per uno stato intermedio in cui si forma Acetaldeide, ma tale reazione è talmente rapida che in pratica l'acetaldeide non ha vita.

Tali sensori hanno sempre all'interno un elemento riscaldante (costituito da una lega di Nickel-Cromo). Questo è necessario per vari motivi. La sensibilità è difatti in funzione della temperatura: maggiore è la temperatura maggiore è la capacità del sistema di "captare" molecole di gas nell'ambiente circostante. Le reazioni che si sviluppano sulla superficie del sensore sono endotermiche, ovvero assorbono calore dall'ambiente circostante, e un aumento di temperatura favorisce il verificarsi delle reazioni chimiche, aumentando di conseguenza la sensibilità, così come l'adsorbimento di ossigeno.

Nell'applicazionesviluppatasiutilizzaunADCperdeterminareladiminuzionediresistenzadelsensoreche ci indica che nell'ambiente circostante è appunto presente alcool.
Un sistema di acquisizione dei dati appositamente costruito si occupa anche di rilevare la temperatura ambiente e l'umidità relativa, in modo da completare le informazioni raccolte.

Schema costruttivo del sensore (fonte: datasheet)



	Parts	Materials
1	Gas sensing layer	SnO_2
2	Electrode	Au
3	Electrode line	Pt
4	Heater coil	Ni-Cr alloy
5	Tubular ceramic	Al_2O_3
6	Anti-explosion network	Stainless steel gauze (SUS316 100-mesh)
7	Clamp ring	Copper plating Ni
8	Resin base	Bakelite
9	Tube Pin	Copper plating Ni

Fig.2

Sensibilità del sensore a vari gas (fonte: datasheet)

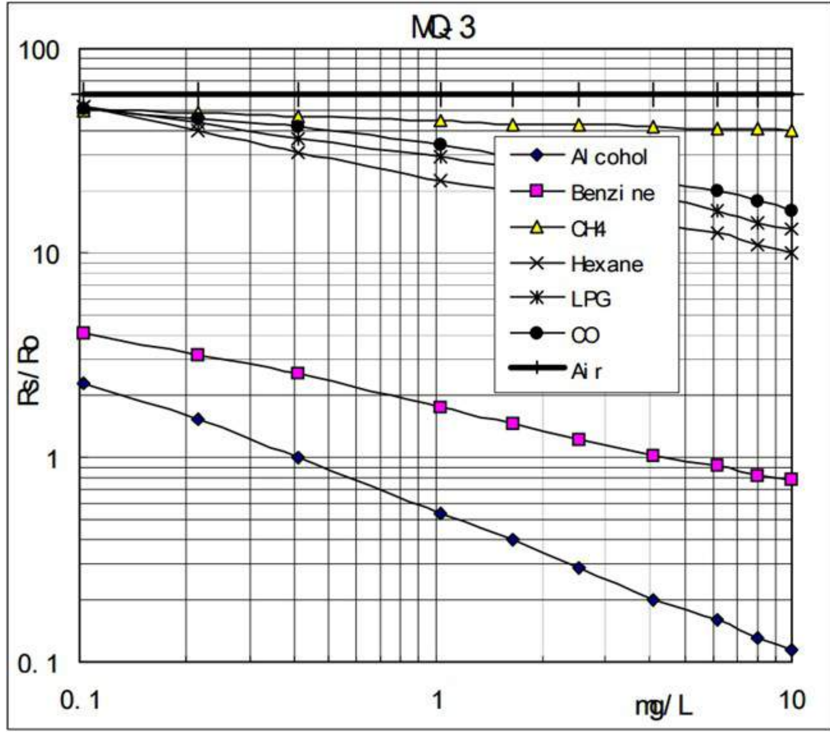


Fig.2 sensitivity characteristics of the MQ-3

Fig.3 is shows the typical sensitivity characteristics of the MQ-3 for several gases.

in their: Temp: 20℃、
Humidity: 65%、
O_2 concentration 21%
RL=200k Ω

Ro: sensor resistance at 0.4mg/L of Alcohol in the clean air.

Rs:sensor resistance at various concentrations of gases.

Relazione tra la resistività e la temperatura ed umidità (fonte: datasheet)

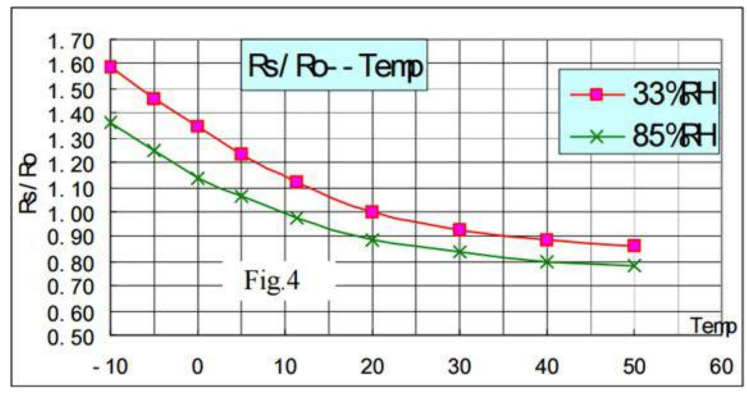


Fig.4 is shows the typical dependence of the MQ-3 on temperature and humidity.

Ro: sensor resistance at 0.4mg/L of Alcohol in air at 33%RH and 20 ℃

Rs: sensor resistance at 0.4mg/L of Alcohol at different temperatures and humidities.

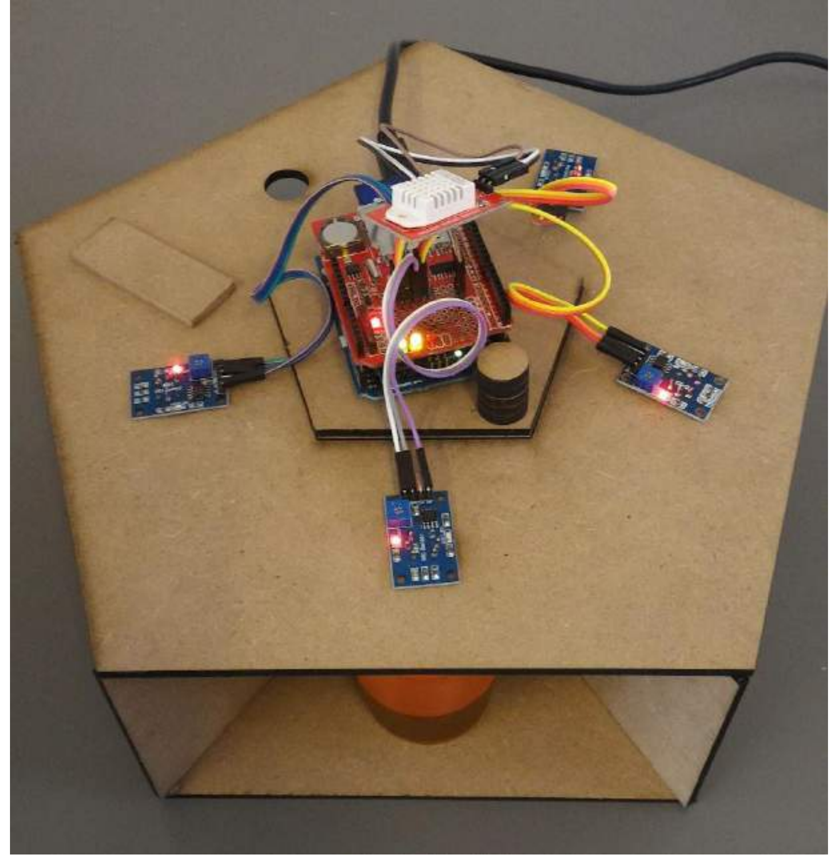
3. TEST E

CONSIDERAZIONI TEST 1:

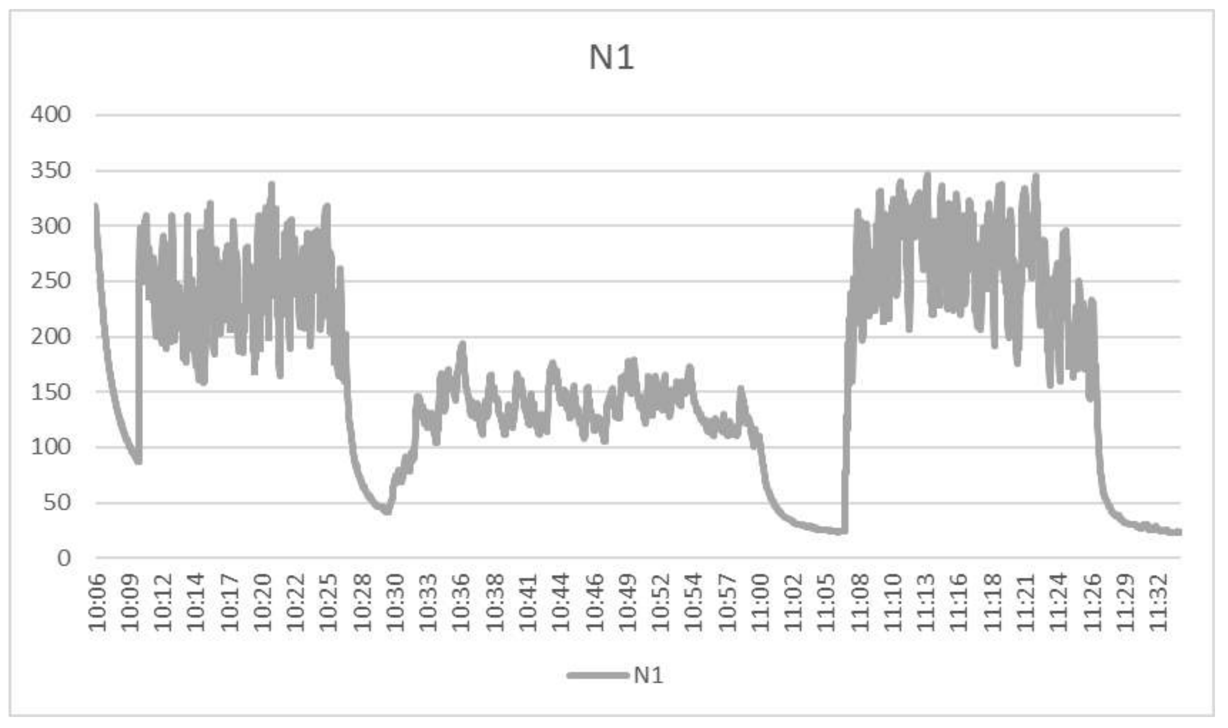
Test eseguito con:

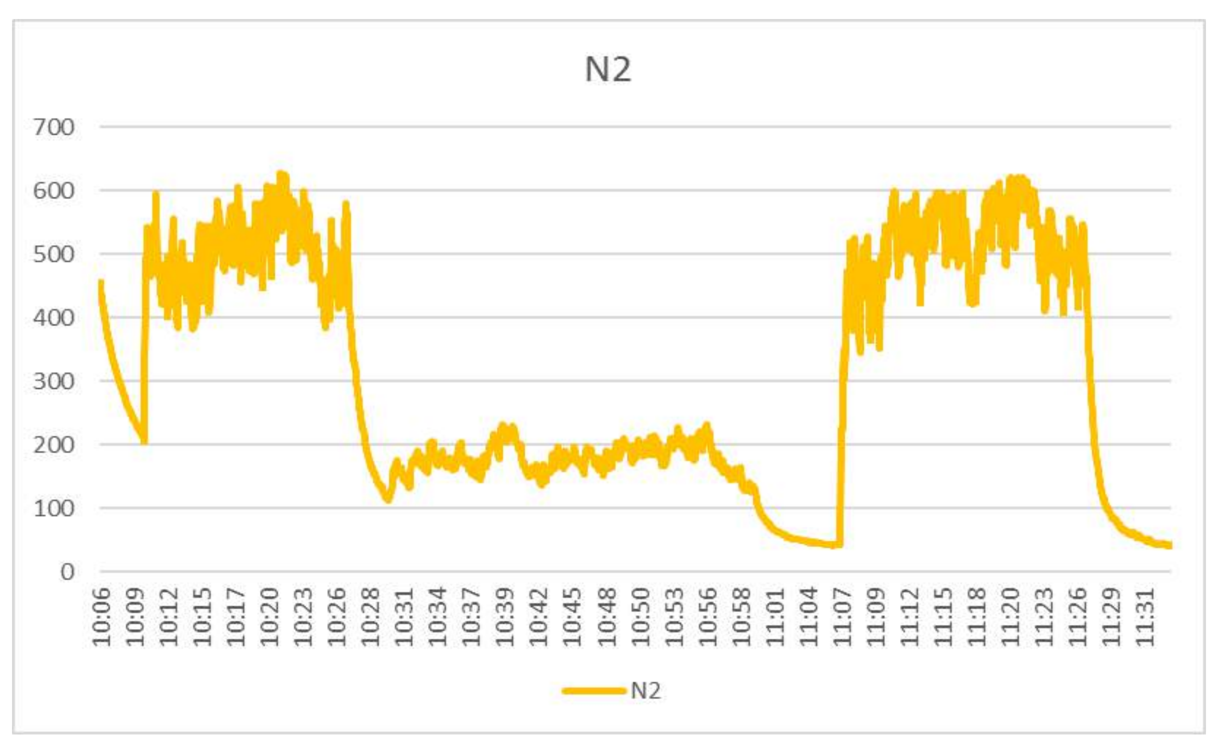
- sensore MQ-3 N1-4
- scheda di acquisizione 10 bit
- intervallo di lettura 1,5Sec
- RTC (real time clock) per TIMESTAMP
- Temp. e Umidità durante il test: 22° - 49% Hr
- Sequenza: VINO, ACETO, VINO IN ACETIFICAZIONE

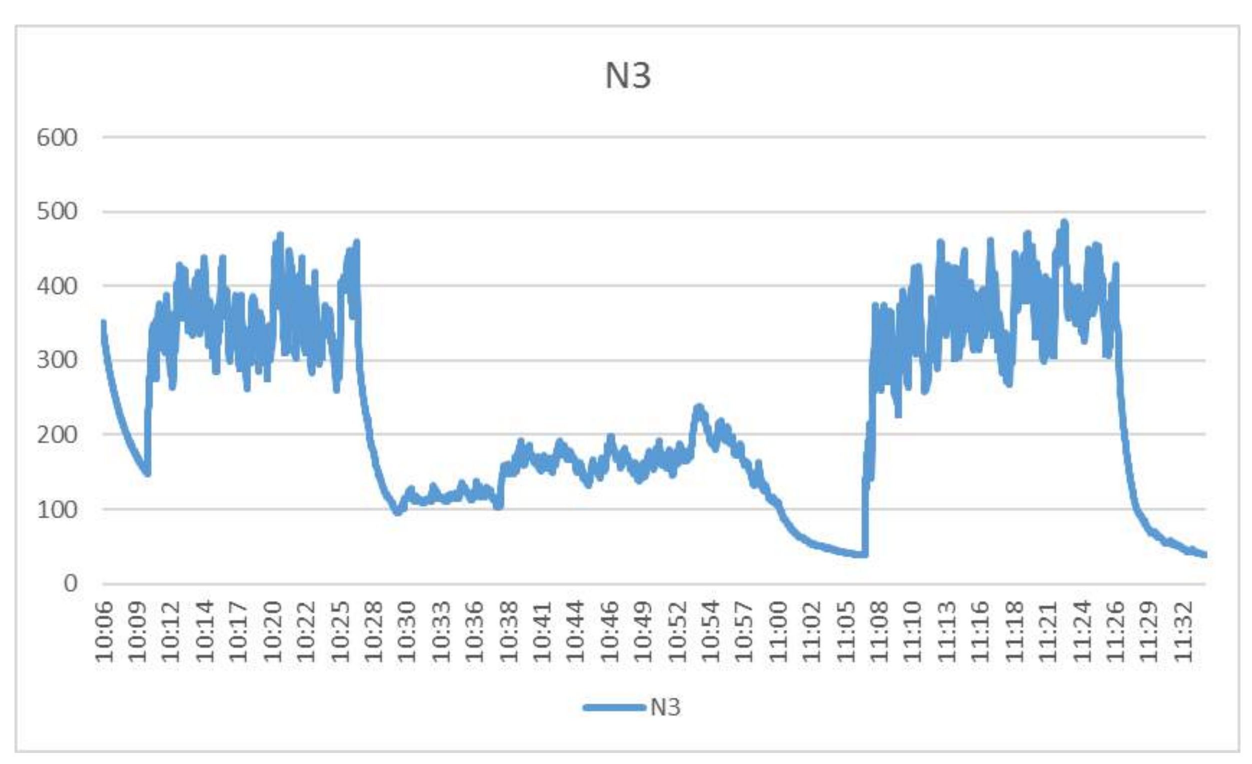
➔ sono stati effettuati n. 4 test, con n. 4 sensori MQ3 diversi per valutare l'affidabilità del sistema.



I risultati sono stati graficati di seguito:






N3
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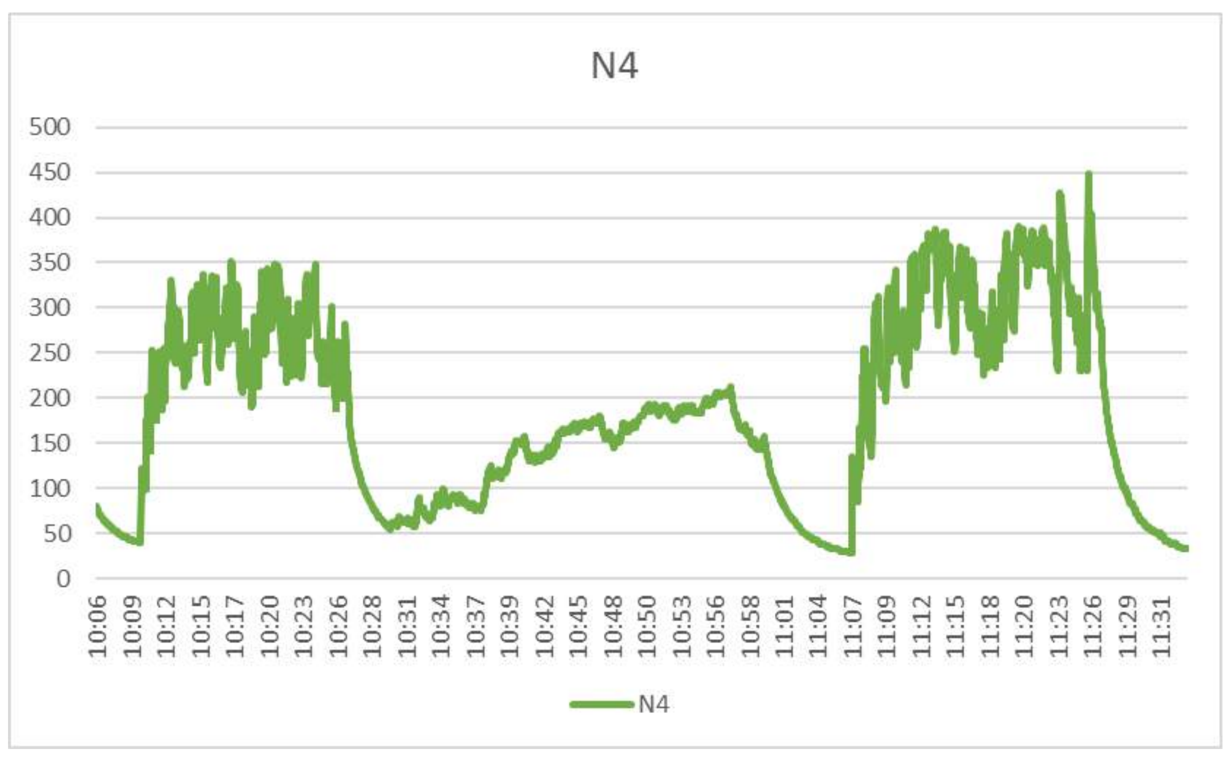
N3


N4
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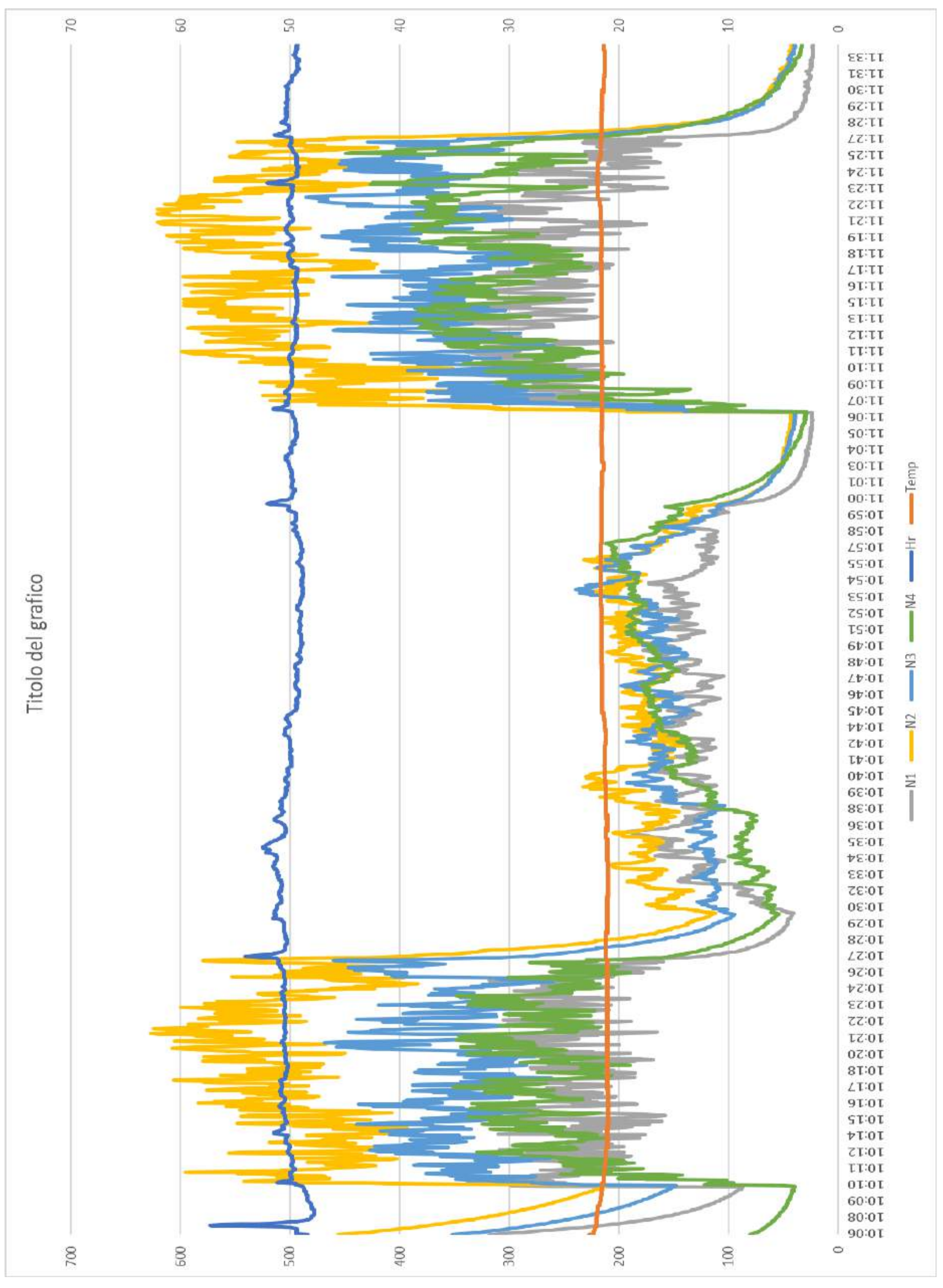
11:31
N4

I risultati sono stati sovrapposti, e si è provveduto ad estrarre anche i dati °T/Hr.

Il risultato è molto più leggibile:



OSSERVAZIONI:

questo primo risultato, comparando i 4 sensori diversi utilizzati, non è molto promettente per le seguenti ragioni:

1. i dati di acquisizione dei singoli sensori sono molto variabili, a parità di condizioni del liquido da rilevare
2. i dati di sensori diversi sono, in molte parti, assolutamente sovrapponibili, segno che l'intervallo di variabilità è troppo alto e di conseguenza i diversi liquidi non si possono sempre distinguere bene.
3. In particolare si nota una certa tendenza, per cui sembra ipotizzabile che il liquido definito "vino in acetificazione" sia stato preso quasi all'inizio del processo, tuttavia alcuni valori massimi di rilevazione di aceto arrivano a toccare i valori minimi delle rilevazioni del vino: questo purtroppo evidenzia i limiti dell'approccio.

TEST 2:

E’ stato preparato ed eseguito un secondo test, cercando in questo caso di eliminare ogni possibile influenza dell’ambiente.

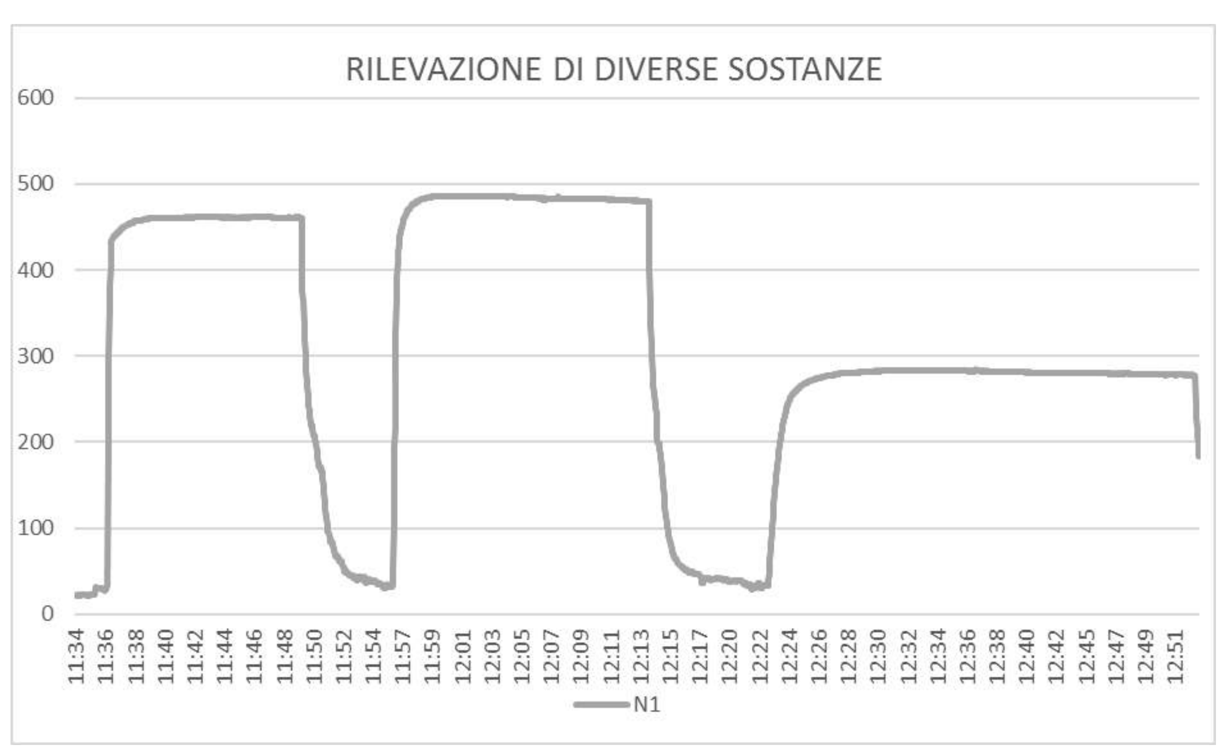
Poiché ciò che viene letto dal sensore è un vapore, una componente gassosa volatile, i bicchieri che contengonoilliquidodurante iltestsonostatiavvicinatiallapareteincuièpresenteilsensore.Inquestoha aiutatoil particolare supporto di test utilizzato, che ha consentito di far si che non ci fosse più aria esterna che potesse disturbare l’evaporazione delle componenti volatili.

I parametri del test sono poi rimasti inalterati, ed anche il nome dei sensori è lo stesso. E’ invece cambiata la sequenza dei liquidi, partendo dal vino in acetificazione per capire se, lasciandolo per ultimo, il sensore soffrisse di una certa “saturazione” e fosse diventato meno preciso nella lettura.

Ecco l’elenco dei parametri di test:

- sensore MQ-3 N1-4
- scheda di acquisizione 10 bit
- intervallo di lettura 1,5Sec
- RTC (real time clock) per TIMESTAMP
- Temp. e Umidità durante il test: 22° - 49% Hr
- Sequenza: VINO IN ACETIFICAZIONE, ACETO, VINO

➔ sono stati effettuati n. 4 test, con n. 4 sensori MQ3 diversi per valutare l’affidabilità del sistema.


RILEVAZIONE DI DIVERSE SOSTANZE
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11:42
11:44
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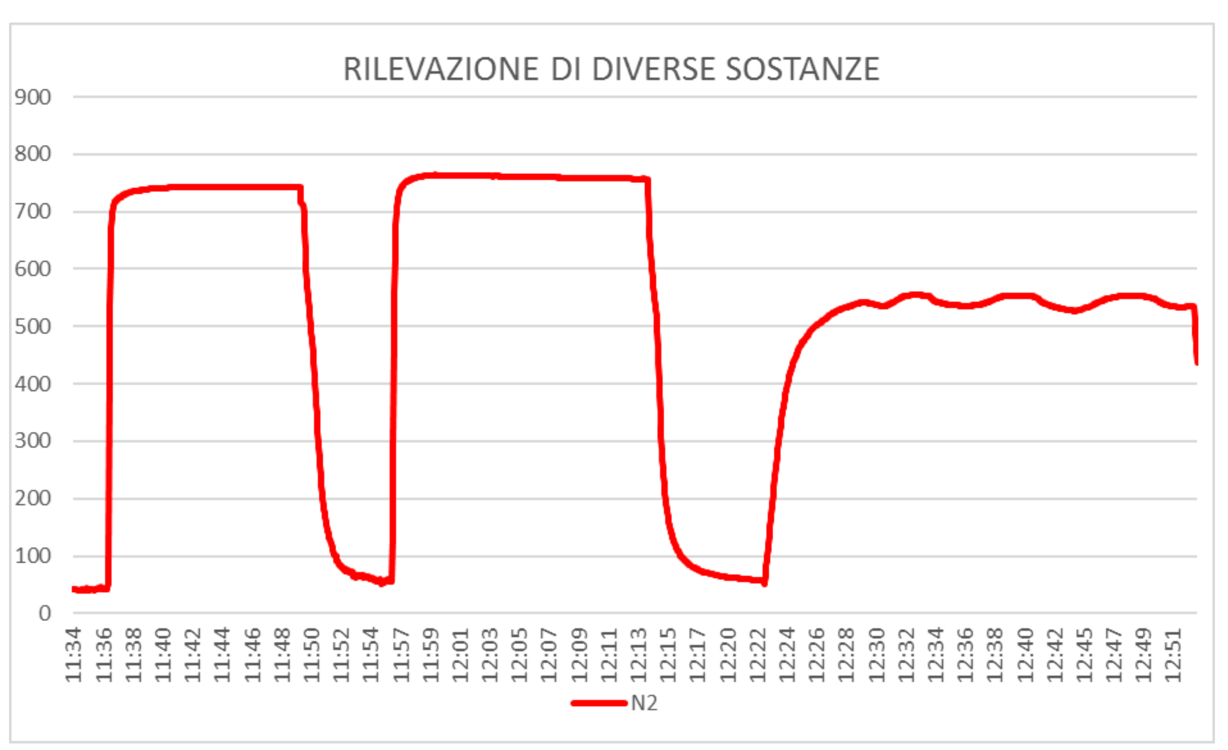
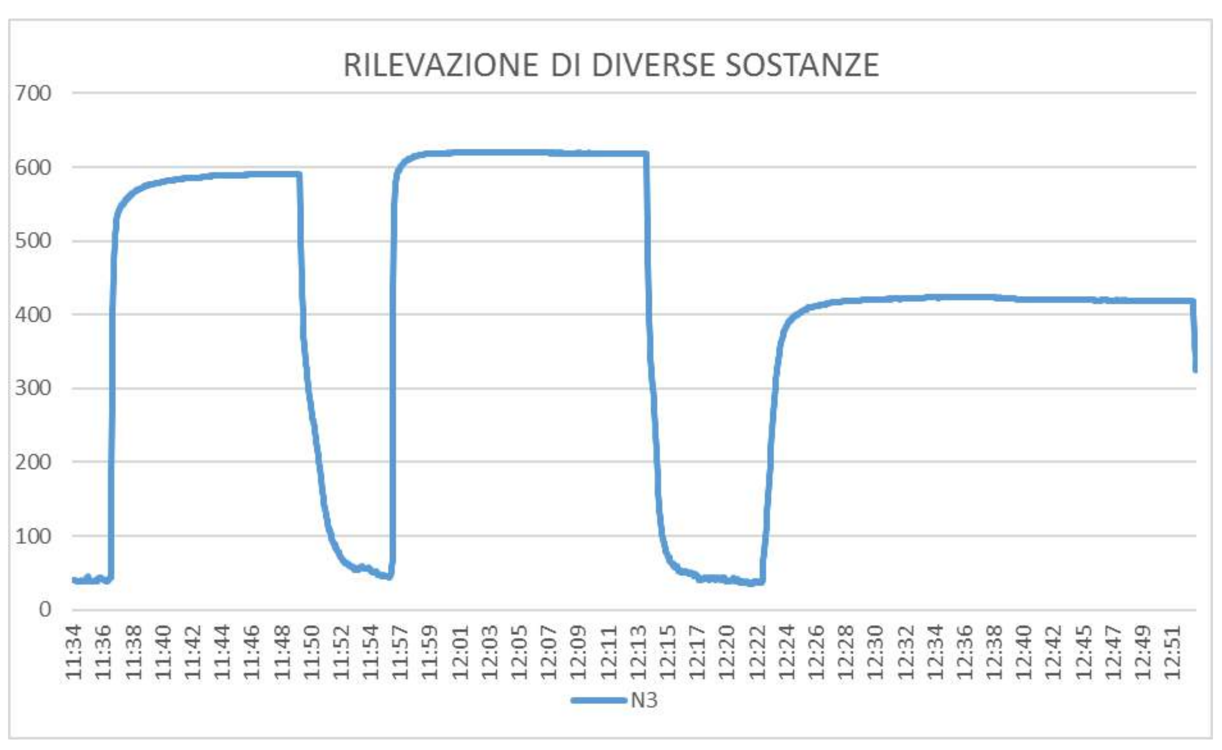
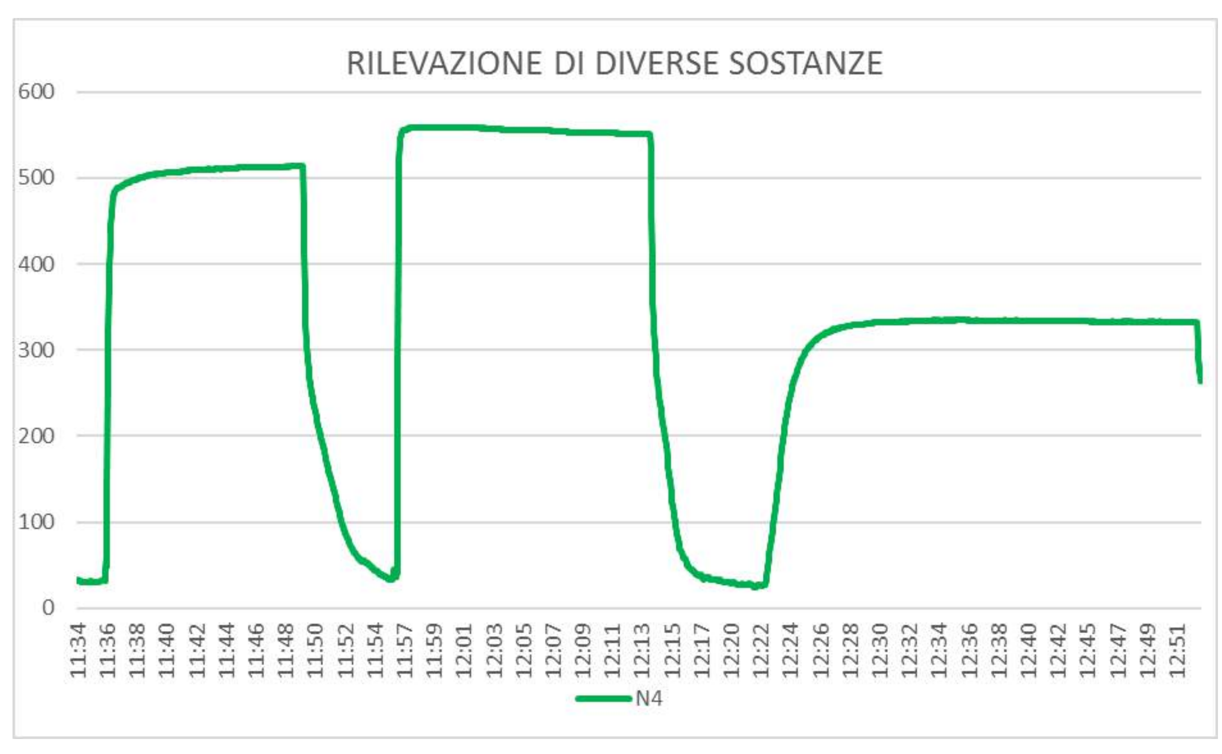
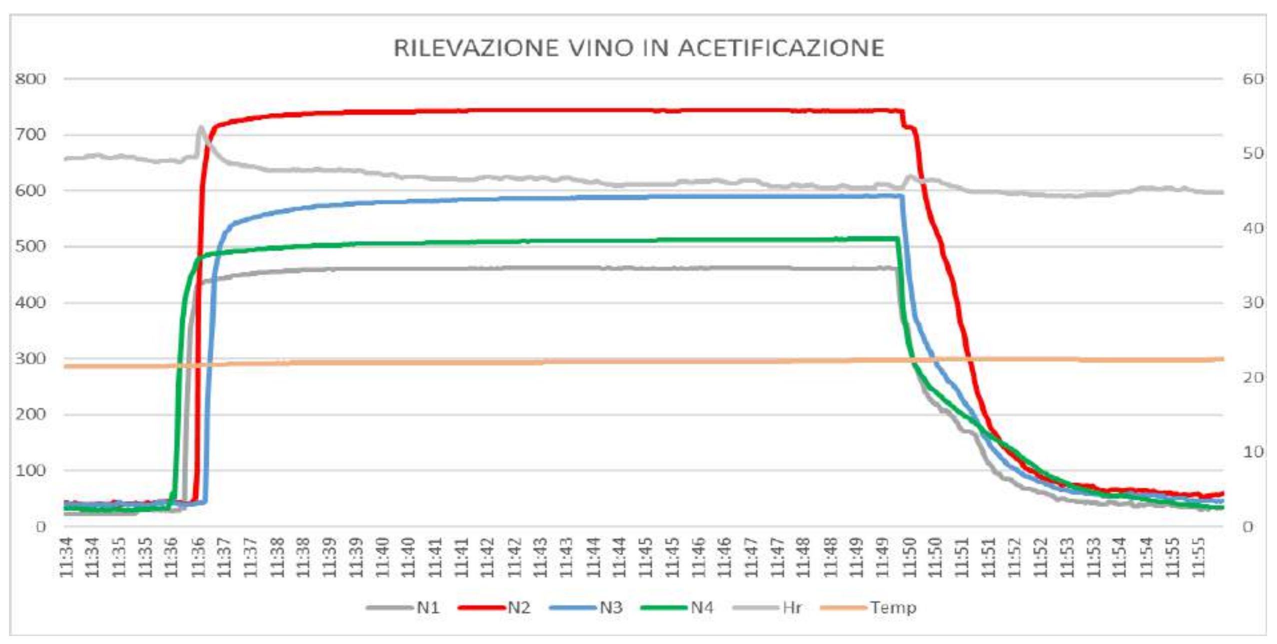
11:48
11:50
11:52
11:54
11:57
11:59
12:01
12:03
12:05
12:07
12:09
12:11
12:13
12:15
12:17
12:20
12:22
12:24
12:26
12:28
12:30
12:32
12:34
12:36
12:38
12:40
12:42
12:45
12:47
12:49
12:51
N1

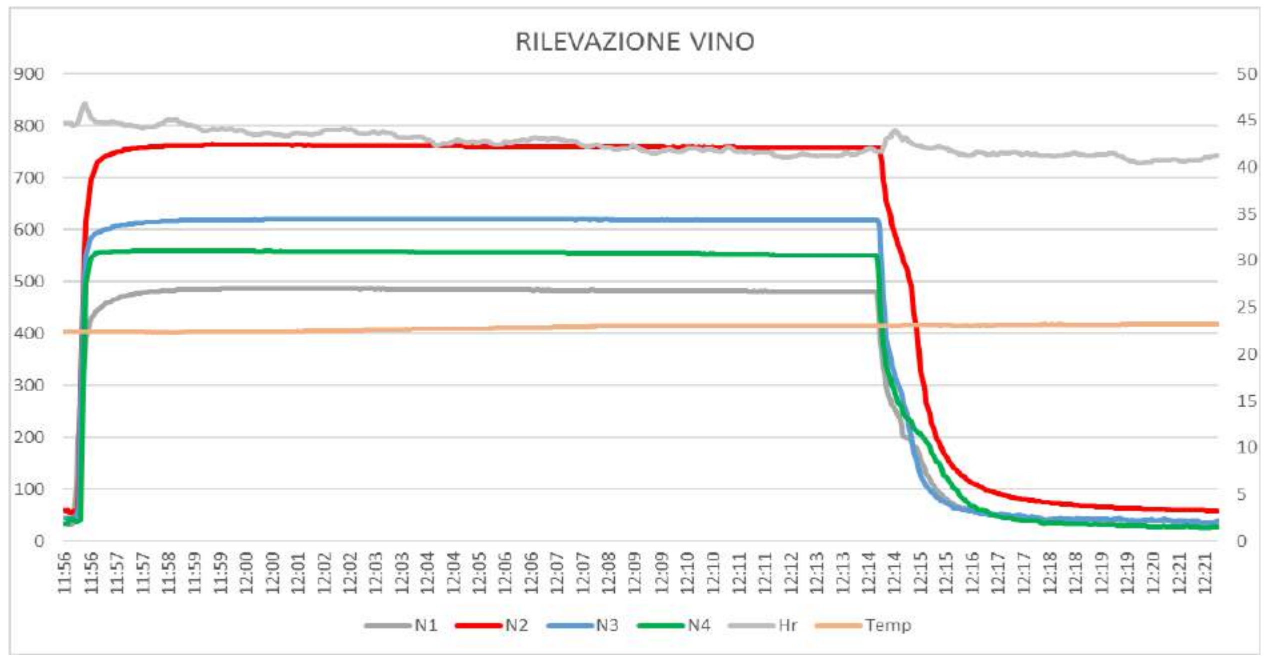

RILEVAZIONE DI DIVERSE SOSTANZE
900
800
700
600
500
400
300
200
100
0
11:34
11:36
11:38
11:40
11:42
11:44
11:46
11:48
11:50
11:52
11:54
11:57
11:59
12:01
12:03
12:05
12:07
12:09
12:11
12:13
12:15
12:17
12:20
12:22
12:24
12:26
12:28
12:30
12:32
12:34
12:36
12:38
12:40
12:42
12:45
12:47
12:49
12:51
N2

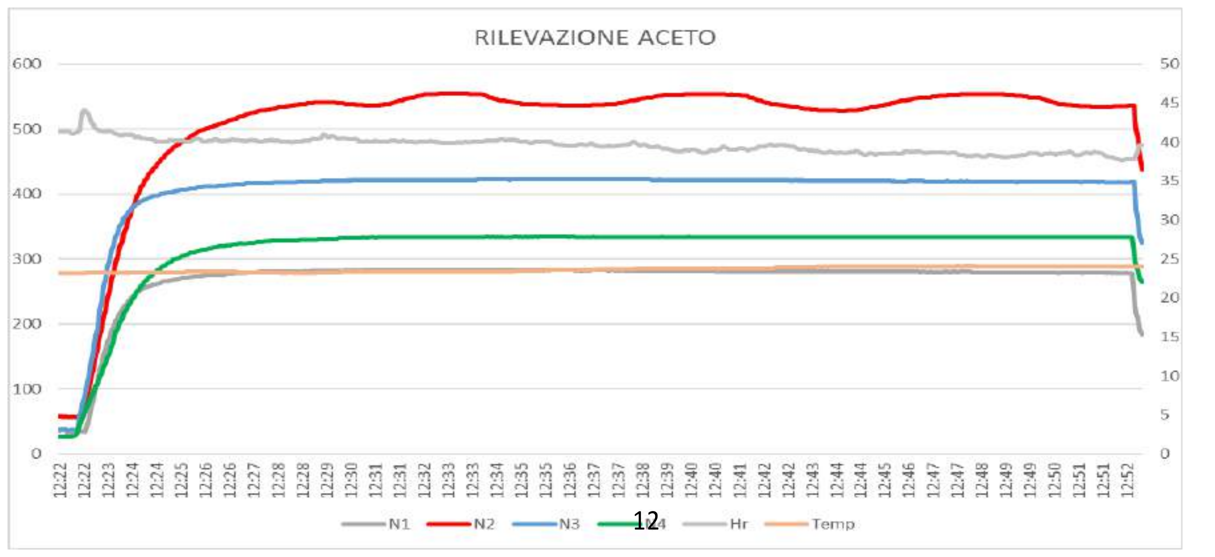

RILEVAZIONE DI DIVERSE SOSTANZE
700
600
500
400
300
200
100
0
11:34
11:36
11:38
11:40
11:42
11:44
11:46
11:48
11:50
11:52
11:54
11:57
11:59
12:01
12:03
12:05
12:07
12:09
12:11
12:13
12:15
12:17
12:20
12:22
12:24
12:26
12:28
12:30
12:32
12:34
12:36
12:38
12:40
12:42
12:45
12:47
12:49
12:51
N3

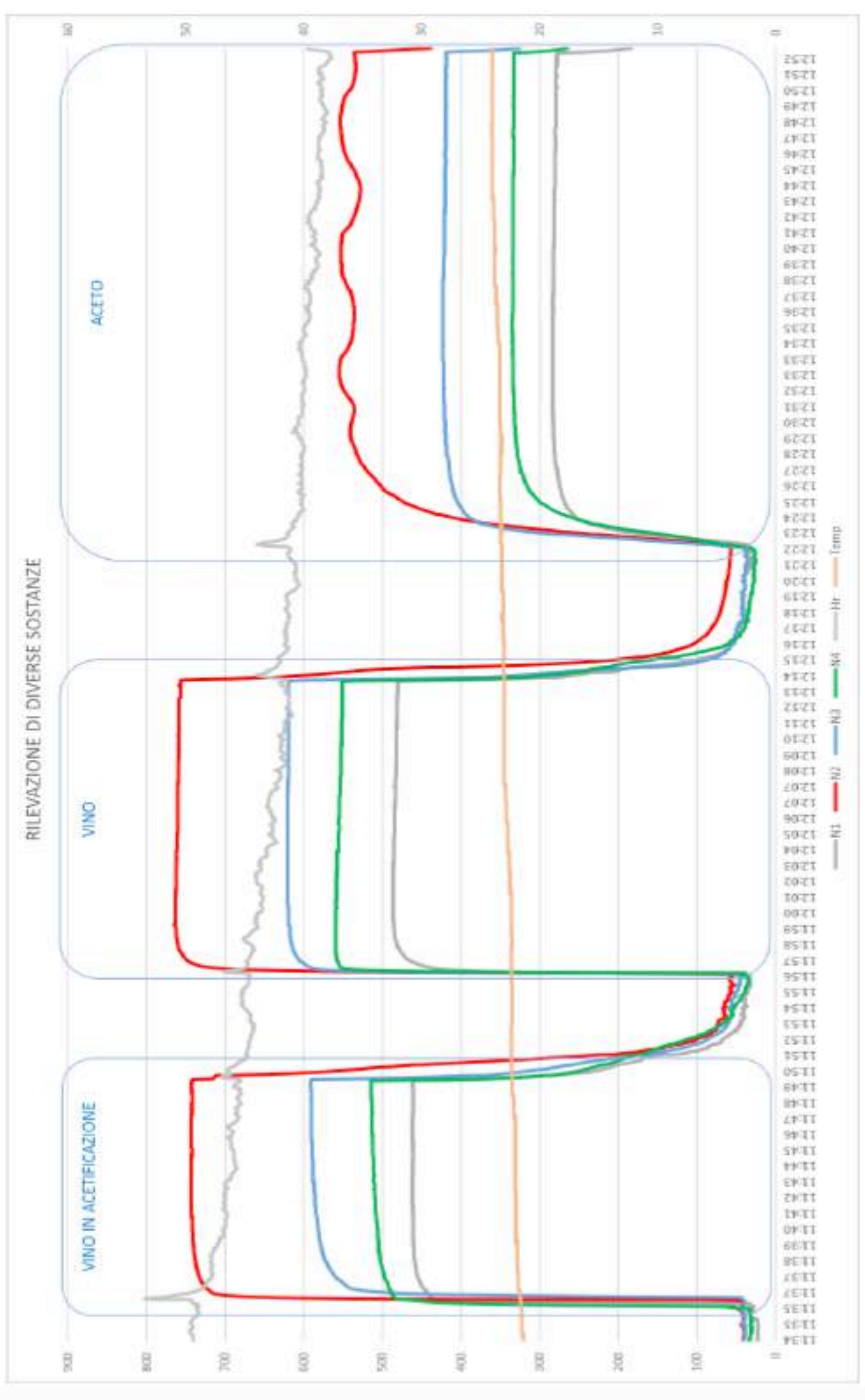

RILEVAZIONE DI DIVERSE SOSTANZE
600
500
400
300
200
100
0
11:34
11:36
11:38
11:40
11:42
11:44
11:46
11:48
11:50
11:52
11:54
11:57
11:59
12:01
12:03
12:05
12:07
12:09
12:11
12:13
12:15
12:17
12:20
12:22
12:24
12:26
12:28
12:30
12:32
12:34
12:36
12:38
12:40
12:42
12:45
12:47
12:49
12:51
N4

I dati raccolti sono stati graficati insieme, ed il risultato è molto più promettente:








RILEVAZIONE DI DIVERSE SOSTANZE
VINO IN ACETIFICAZIONE
VINO
ACETO
N1
N2
N3
N4
Hr
Temp

Analizzando gli esiti della raccolta dati, e in particolare analizzando l'ultimo grafico in cui i 4 sensori sono stati rappresentati insieme su un unico grafico che comprende le 3 prove fatte (analisi di VINO in ACETIFICAZIONE, VINO e ACETO in quest'ordine) è possibile fare qualche considerazione:

- i sensori hanno funzionato in modo atteso in questa modalità di test (senza aria esterna) indicando che è corretta la strada seguita di creare un ambiente di test isolato dall'ambiente esterno, in cui i sensori e l'aria non subiscano interferenze, correnti etc
- i valori raccolti sono COERENTI, ossia tutti i sensori utilizzati leggono alti valori di alcol etilico nel VINO, bassi valori nell'ACETO
- i sensori hanno una sensibilità che può variare, ma resta coerente in tutte le letture (ossia il sensore N2 risulta essere il più sensibile, e questo è costante con tutti i 3 diversi liquidi testati
- i sensori non vanno in "saturazione" anche se lasciati a lungo in una atmosfera SATURA: questo possibile comportamento era da verificare puntualmente, perché il funzionamento del sensore poteva lasciare qualche dubbio ed andava quindi testato (il fatto che il metallo costituente l'elemento sensibile sia "poroso" e che l'elemento volatile legandosi ad esso ne cambia la resistività lascia intendere un'usura nel tempo, e la durata non è stata ancora verificata sperimentalmente)
- il sensore ha una dinamica assolutamente adatta all'applicazione ipotizzata (ossia è sufficientemente veloce nel cambiamento di stato sia in presenza del gas, che quando questo viene rimosso)
- i sensori utilizzati hanno un "guadagno" fisso (non identico, poiché i componenti elettronici hanno comunque una loro tolleranza) e quindi il diverso comportamento non è predicibile a priori. Detto in altre parole, è necessario TARARE il sensore utilizzando un liquido campione noto prima di poterlo utilizzare nella rilevazione, se si vuole avere un risultato non solo qualitativo ma anche di misura
- analizzando le letture dei sensori con i diversi liquidi su nota che le letture del VINO IN ACETIFICAZIONE sono prossime ai valori letti per il VINO. Questo potrebbe sicuramente indicare che il liquido prelevato era in uno stadio INIZIALE di fermentazione, ma non dice nulla sul comportamento durante il processo di acetificazione. NON SI SA SE IL PROCESSO EVOLVA in modo LINEARE (ossia se la concentrazione di alcol etilico diminuisca in modo lineare nel tempo) ma è lecito pensare che NON SIA COSi' (anche solo per la difficoltà oggettiva di mantenere perfettamente equilibrate le concertrazioni durante la fermentazione in un tempo di qualche settimana). Su questo è necessario approfondire l'indagine.
- si nota un piccolo valore di "bias" nelle letture a vuoto. Questo valore, in generale basso e facilmente filtrabile, risulta coerente con la sensibilità del singolo sensore e risulta più elevato per il sensore N2 (che è anche il sensore più sensibile del lotto). Questo porta a credere che la fase di CALIBRAZIONE necessaria possa anche normalizzare le letture andando a rilevare il valore a vuoto e eliminandolo dalle successive letture. Oltre a questo, si segnala che il comportamento sopra descritto è normale per vari dispositivi elettronici e quindi in qualche modo atteso. Potrebbe anche trattarsi di RUMORE e non di BIAS: in ogni caso il basso valore rilevato non desta alcuna preoccupazione.
- L'elettronica di condizionamento del sensore può influenzare le letture (per quanto detto sopra) e dovrebbe quindi essere realizzata con un sistema di taratura specifica; oltre a questo, anche la tensione di l'alimentazione risulta critica e dovrebbe essere ben regolata

L'analisi dei RANGE di lettura mostra dati interessanti per valutare la dinamica dei sensori:

TEST CON VINO IN ACETIFICAZIONE

	N1	N2	N3	N4
MIN	22	39	35	25
MED	312	504	387	351
MAX	462	743	591	514
VAR	440	704	556	489

TEST CON VINO

	N1	N2	N3	N4
MIN	29	55	35	25
MED	355	567	451	406
MAX	486	765	620	559
VAR	457	710	585	534

TEST CON ACETO

	N1	N2	N3	N4
MIN	31	52	36	26
MED	268	510	403	314
MAX	284	555	423	335
VAR	253	503	387	309

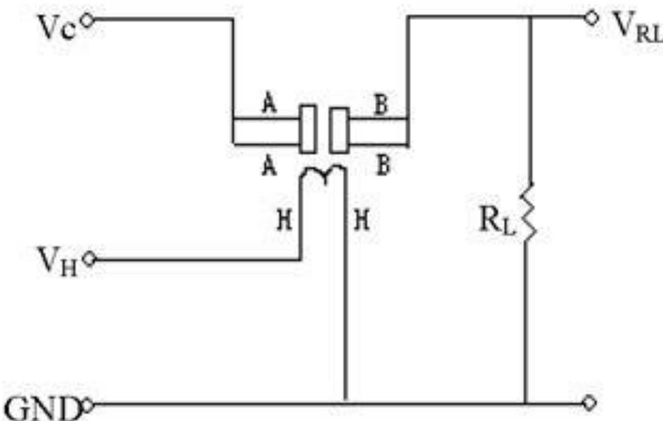
MAX()	N1	N2	N3	N4
ACETO	284	555	423	335
VINO IN AC.	462	743	591	514
VINO	486	765	620	559
SPREAD	**202**	**210**	**197**	**224**

MED()	N1	N2	N3	N4
ACETO	268	510	403	314
VINO IN AC.	312	504	387	351
VINO	355	567	451	406
SPREAD	**86,99**	**57,52**	**47,81**	**92,16**

VAR. MAX	N1	N2	N3	N4
ACETO	253	503	387	309
VINO IN AC.	440	704	556	489
VINO	457	710	585	534
SPREAD	**204**	**207**	**198**	**225**

- Il sensore N2 mostra una variazione maggiore (differenza tra massimo e minimo valore rilevato)
- Il sensore N4 mostra invece la variazione massima in valore assoluto tra le letture dei 3 liquidi
- I dati mostrano un comportamento dei sensori comunque abbastanza omogeneo, a dispetto della rappresentazione nei grafici allegati. Questo significa che la taratura dei sensori dovrebbe consentire di ottenere prestazioni assolute simili e risultati molto soddisfacenti.

ANALISI ELETTRICA:



In figura è rappresentato lo schema elettrico semplificato del sensore, in cui si evidenzia la parte del riscaldatore (che richiede una tensione Vh) e la parte di alimentazione del rilevatore (Vc).

L'uscita del rilevatore (Vrl) vede la necessiatà di applicazione di un carico (Rl) su cui rilevare la corrente che transita all'interno dell'elemento rilevatore, che è direttamente dipendente da Vc e dal valore della resistenza di caduta Rl (che serve in questo caso da semplice convertitore corrente-tensione).

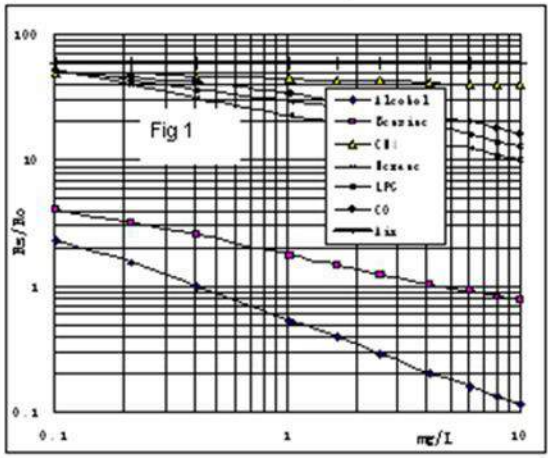
Una possibile calibrazione potrebbe intervenire in quest'ultimo punto, andando a sostituire Rl con un partitore resistivo, composto da 2 resistenze una delle quali variabile con precisione (es. resistore multigiri).

I sensori utilizzati sono già integrati di elettronica, non è quindi stato possibile effettuare regolazioni e quindi su questi aspetti si potrà approfondire successivamente.

ANALISI IN TEMPERATURA

La reazione di fermentazione che avviene è dipendente dalla temperatura, e lo stesso sensore durante il suo funzionamento dipende da TEMPERATURA ed UMIDITA' dell'ambiente. Nel datasheet è indicato il comportamento tipico in funzione di questi 2 parametri:

Sensitivity Characteristics



Influence of Temperature/Humidity

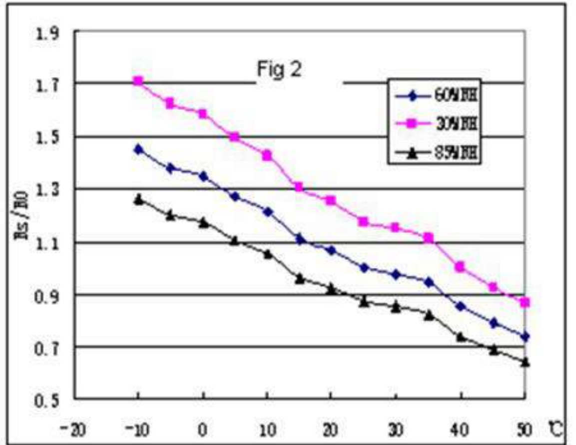
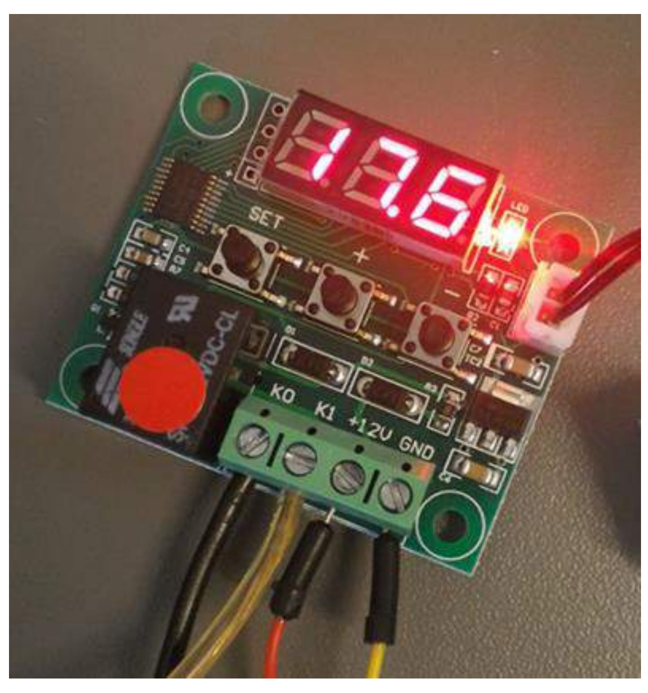


Fig.1 shows the typical sensitivity characteristics of the MQ-3, ordinate means resistance ratio of the sensor (Rs/Ro), abscissa is concentration of gases. Rs means resistance in different gases, Ro means resistance of

Fig.2 shows the typical temperature and humidity characteristics. Ordinate means resistance ratio of the sensor (Rs/Ro), Rs means resistance of sensor in 0.4mg/l alcohol under different tem. and humidity.

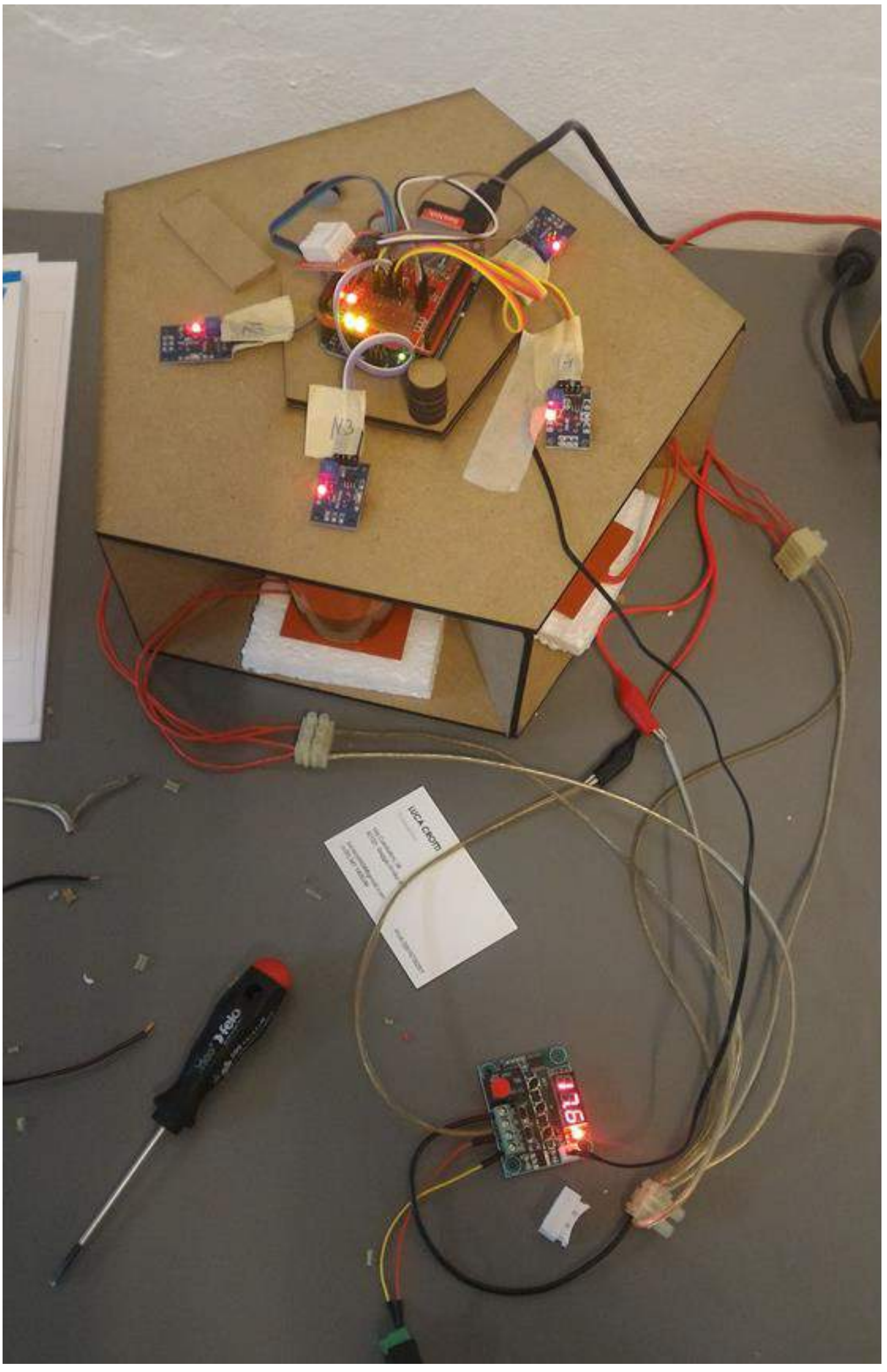
È stato quindi modificato ulteriormente il prototipo di misura realizzato, aggiungendo un riscaldatore per ogni gruppo sensore+liquido.

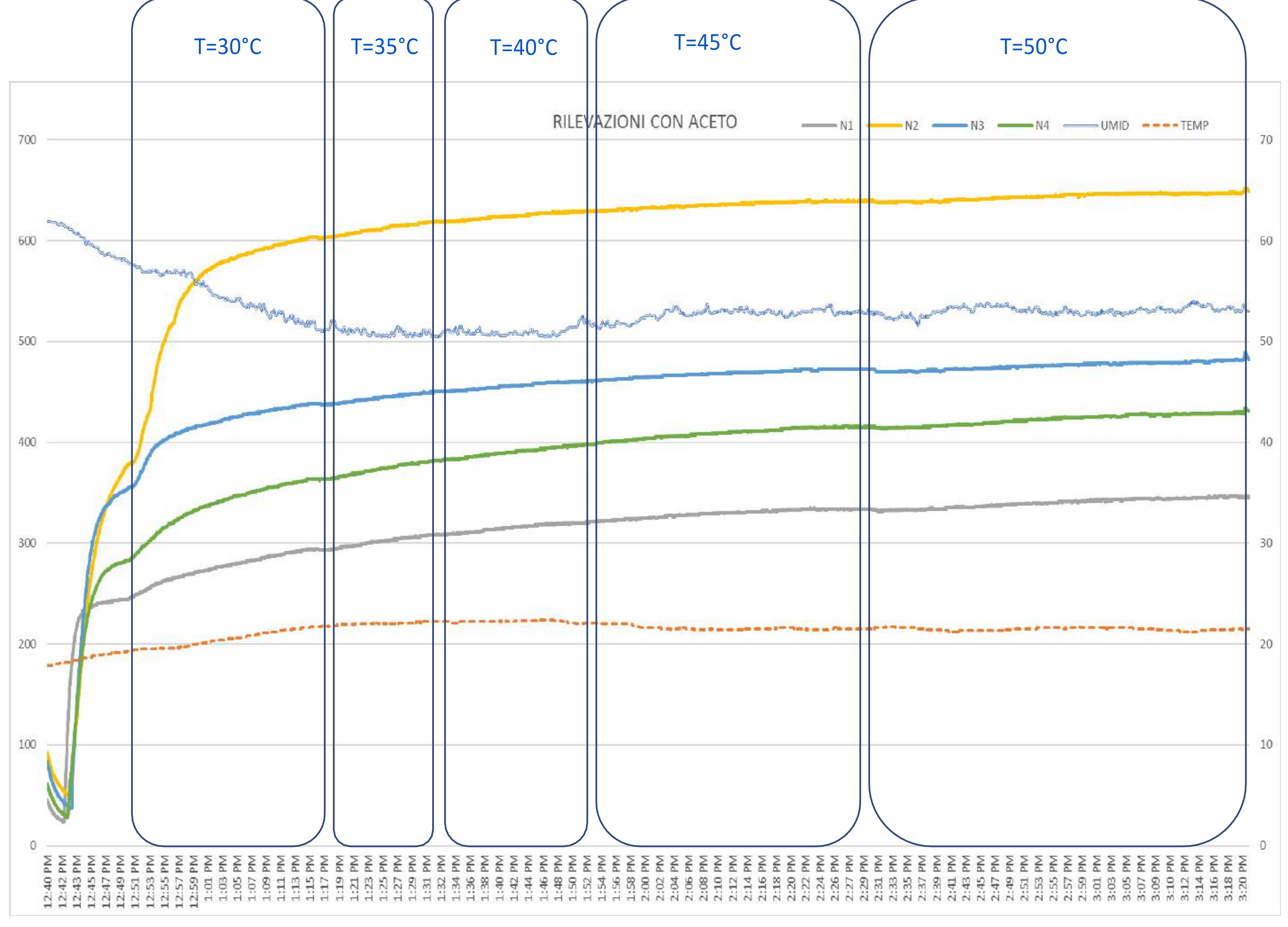


Il riscaldatore è controllato utilizzando un circuito di termostatazione composto da un sensore di temperatura analogico racchiuso in un involucro metallico per renderlo resistente all'immersione nel liquido di test.

La temperatura è stata regolata con una precisione di 0.5°C.

Nell’immagine seguente è rappresentato il banco di test modificato:




T=30°C
T=35°C
T=40°C
T=45°C
T=50°C
RILEVAZIONI CON ACETO
N1
N2
N3
N4
UMID
TEMP
700
600
500
400
300
200
100
0
70
60
50
40
30
20
10
0
12:40 PM 12:42 PM 12:43 PM 12:45 PM 12:47 PM 12:49 PM 12:51 PM 12:53 PM 12:55 PM 12:57 PM 12:59 PM 1:01 PM 1:03 PM 1:05 PM 1:07 PM 1:09 PM 1:11 PM 1:13 PM 1:15 PM 1:17 PM 1:19 PM 1:21 PM 1:23 PM 1:25 PM 1:27 PM 1:29 PM 1:31 PM 1:32 PM 1:34 PM 1:36 PM 1:38 PM 1:40 PM 1:42 PM 1:44 PM 1:46 PM 1:48 PM 1:50 PM 1:52 PM 1:54 PM 1:56 PM 1:58 PM 2:00 PM 2:02 PM 2:04 PM 2:06 PM 2:08 PM 2:10 PM 2:12 PM 2:14 PM 2:16 PM 2:18 PM 2:20 PM 2:22 PM 2:24 PM 2:26 PM 2:27 PM 2:29 PM 2:31 PM 2:33 PM 2:35 PM 2:37 PM 2:39 PM 2:41 PM 2:43 PM 2:45 PM 2:47 PM 2:49 PM 2:51 PM 2:53 PM 2:55 PM 2:57 PM 2:59 PM 3:01 PM 3:03 PM 3:05 PM 3:07 PM 3:09 PM 3:10 PM 3:12 PM 3:14 PM 3:16 PM 3:18 PM 3:20 PM

Osservazioni sul test termostatato in temperatura:

- Il test è stato eseguito utilizzato ACETO, liquido in cui la lettura è risultata minore
- Il sistema di riscaldamento ha lavorato in modo efficace, riuscendo a portare il liquido da circa 20°C a 50°C con intervalli di 5°C
- La temperatura ambiente e l'umidità sono state costantemente monitorate, mentre il riscaldatore è stato pilotato in modo manuale
- A temperature ambiente (circa 20°C) le letture erano:
  - N1 → 280
  - N2 → 530
  - N3 → 420
  - N4 → 330
- Le indicazioni della primissima parte del grafico concordano con le letture a 20°C e, in un certo qual modo, sono coerenti con le letture alla temperatura di 30°C
- L'evoluzione delle temperature mostra una crescita costante e coerente: in particolare

ACETO	LETTURE			
T amb	N1	N2	N3	N4
23,6	282	536	423	334
30	290	598	434	359
35	308	619	450	382
40	321	629	461	398
45	334	639	472	415
50	346	648	481	429

Le variazioni, in termini %, sono più significative soprattutto nel primo range di temperatura (da 20 a 30°C); successivamente le variazioni sono costanti anche se contenute. Vista la tendenza, si può ipotizzare che oltre i 60-70°C non ci siano più variazioni nelle letture al variare della temperatura.

I dettagli sono riassunti nelle tabelle sottostanti:

ACETO	DELTA			
T amb	N1	N2	N3	N4
23,6	282	536	423	334
30	8	62	11	25
35	18	21	16	23
40	13	10	11	16
45	13	10	11	17
50	12	9	9	14

ACETO	DELTA %			
T amb	N1	N2	N3	N4
23,6	282	536	423	334
30	2,8%	11,6%	2,6%	7,5%
35	6,2%	3,5%	3,7%	6,4%
40	4,2%	1,6%	2,4%	4,2%
45	4,0%	1,6%	2,4%	4,3%
50	3,6%	1,4%	1,9%	3,4%

Più interessante è l'analisi delle variazioni cumulative, che mostrano una differenza totale nelle letture di un valore complessivo superiore al 20%:

ACETO	DELTA CUMULATIVO			
T amb	N1	N2	N3	N4
23,6	282	536	423	334
30	8	62	11	25
35	26	83	27	48
40	39	93	38	64
45	52	103	49	81
50	64	112	58	95

ACETO	DELTA % CUMULATIVO			
T amb	N1	N2	N3	N4
23,6	282	536	423	334
30	2,8%	11,6%	2,6%	7,5%
35	9,2%	15,5%	6,4%	14,4%
40	13,8%	17,4%	9,0%	19,2%
45	18,4%	19,2%	11,6%	24,3%
50	22,7%	20,9%	13,7%	28,4%

In particolare si conferma l'ottimo comportamento del sensore N2, mentre appare chiaro che il sensore N3 ed il sensore N4 (pure interessante nei test precedenti) si comportano in modo diverso. Questi 2 sensori potrebbero essere considerati gli estremi della variabilità, considerando quindi la dipendenza dalla temperatura compresa nel range 14-28%

Un'altra caratteristiche che emerge è la non perfetta linearità dei sensori: se si osserva l'andamento dei sensori N1 ed N2, per esempio, si nota come il secondo (N2) abbia dato il 75% della sua variazione totale già alla temperatura di 35°C, mentre per il sensore N1 la variazione si attesta al di sotto del 50% della variazione totale.

Analizzando tutti i sensori in esame, il comportamento tipico sembra essere più simile a quello mostrato dal sensore N1 e più in generale si può dire che già a 35°C i sensori incrementano del 50% le letture rispetto al totale dell'incremento nel range considerato.

Un'osservazione più generale (che bisognerebbe però verificare sperimentalmente) potrebbe quindi essere quella relativa alla variazione complessiva del range di lettura: le letture diventano più affidabili a temperature superiori, mentre a temperature più basse i sensori non sono così "selettivi" o, per meglio dire, così precisi.

Un'altra osservazione riguarda i range di lettura, e più in generale lo scostamento dei 4 sensori. In particolare, il sensore N2 è uno dei sensori con più dinamica ma allo stesso tempo è il sensore con letture più distanti dagli altri. Infatti, eliminando il sensore N2 dalle tabelle, si nota come il range risulti più simile:

ACETO	LETTURE			
T amb	N1	N2	N3	N4
23,6	282	536	423	334
30	290	598	434	359
35	308	619	450	382
40	321	629	461	398
45	334	639	472	415
50	346	648	481	429

ACETO	RANGE			
T amb	range min - max		media	Delta
23,6	282	536	393,75	254
30	290	598	420,25	308
35	308	619	439,75	311
40	321	629	452,25	308
45	334	639	465	305
50	346	648	476	302

ACETO	RANGE - senza N2			
T amb	range min - max		media	Delta
23,6	282	423	346,3333	141
30	290	434	361	144
35	308	450	380	142
40	321	461	393,3333	140
45	334	472	407	138
50	346	481	418,6667	135

Questo, ancora una volta, è ipotizzabile che sia dovuto alla variabilità dei materiali dell’elemento sensibile ed anche alle tolleranze dei componenti discreti utilizzati, soprattutto della resistenza di caduta della tensione (infatti la tensione era uguale per tutti i sensori). Questo rafforza l’idea che si debba realizzare un circuito di pilotaggio che richieda una taratura del sensore prima di poterlo utilizzare per misure affidabili.

4. CONCLUSIONI

Le analisi effettuate fino ad oggi hanno potuto sperimentare diverse tecnologie, diversi sensori ed anche il loro comportamento dinamico (cioè nel tempo) al variare della temperatura ambientale e del liquido di test. Queste le conclusioni sintetiche del lavoro svolto:

- ❖ I sensori utilizzati sono in grado di discriminare la presenza e la concentrazione di alcol etilico e, soprattutto, monitorare la sua variazione nel processo di acetificazione
- ❖ La variabile temperatura gioca un ruolo importante, e rende le letture più robuste soprattutto alle alte temperature, che sono quelle alle quali avviene nella pratica la fermentazione
- ❖ I sensori richiedono una taratura iniziale per poter essere adoperati con successo, ma questa risulta abbastanza semplice da implementare (1 sola taratura in fabbrica)
- ❖ I sensori discriminano bene le concentrazioni di alcol etilico e quindi sono compatibili con il processo di acetificazione sotto esame.

I prossimi passi possibili sono:

- Realizzazione di un circuito dedicato all'applicazione, che consenta il controllo della temperatura e la taratura dei sensori
- Analisi completa durante un vero processo di acetificazione, con l'obiettivo di verificare le ipotesi fatte in queste pagine
- Realizzazione di una mappa di correlazione tra l'evoluzione del processo di acetificazione e il corrispondente livello di alcol etilico (rispetto al valore iniziale), in modo da poter monitorare e quindi verificare il processo di acetificazione in un prodotto commerciale, che possa fornire indicazioni attendibili circa il buon andamento del processo, la coerenza delle letture, la conclusione positiva o negativa del processo di acetificazione (inclusi allarmi intermedi)

Ing. Luca Crotti

Appendix 8.
Kit for domestic production of vinegar

Customer's test
Ristorante Reale (ITALY) – 3 * stars**
Ranked as best Italian restaurant
Started on march 18th, 2016 with company's starter cultures.
On October 10th, 2019 implementing the basic knowledge and control tools, the biofilm is still present. Repeated batch process and scaling-up were autonomously performed.

March, 2016

October, 2019

Appendix 9.
Kit for domestic production of vinegar

Effects of different lyophilisation media on the viability of freeze-dried *Acetobacter pasteurianus* strain AB0220 suitable for vinegar fermentation

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Introduction

Acetobacter pasteurianus has been widely used to produce vinegar in the food industry. However, many strains are difficult to preserve especially those isolated from industrial acetification systems that are high acetic acid (AcOH) tolerant (Kittelman et al., 1989).
Therefore, there is a need to preserve *A. pasteurianus* strains overcoming the instability of key traits, such as ethanol (EtOH) oxidation and AcOH resistance.
Generally, Acetic acid bacteria (AAB) preservation can be performed by short time and long time methods. By short time preservation, cultures are maintained generally at 4 °C and refreshed periodically on new medium. Therefore, they are cyclically on growing status with high probability of mutations inducing phenotypic changes (Gherna and Reddy 2007). To preserve phenotypic and genotypic traits of industrial strains, long time preservation is conducted by freeze-drying, ultra-freezing temperature (-80°C or -150°C) of both pure and starter cultures.
In particular, freeze-drying is one of the most widely used methods for microbial long-term preservation. Cultures generally have good viability/stability and can be stored for many years. Ampoules take up little space and can be easily stored. However, the method shows also some disadvantages with some isolates which fail to survive the process or have reduced viability and may show genetic change. Moreover, cell damage can occur during the cooling and/or drying stages because of the phase changes that can disrupt the fluid-mosaic structure of the membrane. To avoid this, suitable protective agents need to be tested at different concentrations in order to improve strain viability. Among the AAB selected as starter culture for vinegar production, the *A. pasteurianus* strain AB0220 has been successfully applied (Gullo et al. 2012).
In the frame of this PhD thesis, with regards of define a set of procedure and tools for domestic production of vinegars, the possibility to equip the kit with a lyophilized selected starter culture has great importance .

The aim of this investigation was to improve the viability of this strain after freeze-drying and evaluate the effect of the protective lyophilisation medium used. Moreover, the maintenance of the key traits related to EtOH oxidation and AcOH resistance, was assessed in wine after the rehydration of the freeze-dried AB0220 strain.

Materials and Methods

Growth condition of AB0220 strain

The AB0220 *A. pasteurianus* strain belongs to Unimore Microbial Culture Collection (UMCC). The strain was grown in GEY medium agar, composed of 2% D-glucose; 0.8% yeast extract; 0.5% ethanol; 0.5% peptone; 0.3% CaCO3 and 1.5% agar (Yamada et al., 1999).

Lyophilisation medium

Two lyoprotectans, namely mannitol and skim milk, were used for the freeze-dried process. In particular, the skim milk solution 20% (w/v) was prepared and sterilised in autoclave at 121°C for 5 minutes. The mannitol solution 20% (w/v) was sterilised by filtration using cellulose acetate membrane filters (0.2-micron pore size).

Preparation of the cultures for the lyophilisation

Fresh AB0220 cultures, grown on GEY agar plates, were withdrew with a sterile loop and used for inoculation of 1 mL of skim milk solution 20% and 1 mL of mannitol solution 20% in sterile tubes. The two inoculated lyophilisation media were used to fill sterile glass ampoules (200 µl for each ampoule).

Totally 10 ampoules were prepared and before starting the freeze-drying process, all of them. Afterwards, they were put at -80 °C for two hours.

The frozen samples were lyophilised at -50°C and 0,108 millibars in a freeze dryer 5Pascal for 6 hours (Figs 1 and 2). After the ampoules were sealed with a hand torch (Fig.3) and stored in a refrigerator. One ampoule with AB0220 + skim milk (AB0220-sm) and one ampoule with AB0220 + mannitol (AB0220-m) were kept at 37°C for 4 days in order to achieve an accelerated storage, corresponding to a period of 6 years at room temperatures (Sakane 1997).

Fig. 1 Freeze Dryer 5Pascal LIO 5 P SERIES

Fig. 2. Ampoules located inside the freeze-dryer chamber

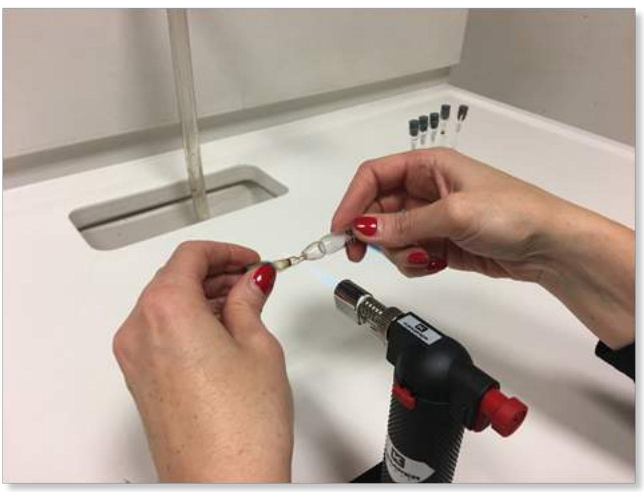


Fig. 3. Ampoules sealed with a hand torch

Cell counting and viability assessment

In order to assess the viability of AB0220, the cells concentration inside the ampoules was estimated by plate count method, before and after the lyophilisation. In particular, 10 fold dilutions of the starting inoculated medium (skim milk or mannitol solution) or the rehydrated culture were made from 10^{-1} to 10^{-9} and of each dilution, 100 µL was transferred and spread to GEY agar plates. The plates were incubated for 4 days at 28°C. The cells concentration was expressed as the colony forming unit for mL (cfu/mL).

After the freeze-dried process, the cells in the ampoules were rehydrated with 200 µl of GYE medium. Viability on GEY plates was determined as previously described. The viability percentage was assessed according the following formula:

$$= \left[\frac{\mathrm{LOG_{10}\ (cfu/ml)\ after\ freeze\text{-}drying,\ base}}{\mathrm{LOG_{10}(cfu/ml)\ before\ freeze\text{-}drying}}\right] \times 100;$$

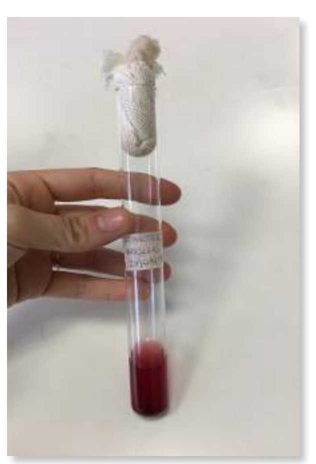
Residual moisture content determination

Residual moisture content (RMC) was obtained by weighted the opened ampoules with freeze-dried samples inside. In particular, after the freeze-drying, two ampoules with AB0220-sm and two ampoules with AB0220-m were opened and weighted by using an analytical balance. Then, the ampoules were incubated overnight in an oven at 150 °C, in order to evaporate residual water, and weighted again at the end.

RMC was determined according the following formula: $((\mathrm{weight}_{\mathrm{initial}} - \mathrm{weight}_{\mathrm{final}})/\mathrm{weight}_{\mathrm{initial}}) \times 100$.

Starter culture preparation

After the freeze-dried process, one ampoule of AB0220-sm and one of AB0220-m were opened and the cell rehydrated with 1,8 mL of sterile wine in tube. The tubes were incubated at 28°C for 48 hours. A scaling-up of the two cultures was performed each two days by adding increasing volumes of sterile wine. After 7 days a final volume of 45 mL for each starter cultures was reached in sterile flasks (Fig. 3). Finally, the titratable acidity was detected.



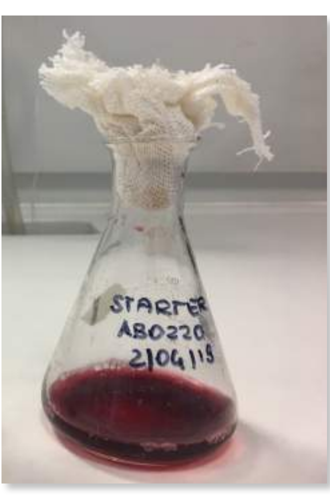


Fig. 3. Starter culture preparation

Results

Effects of different lyoprotectant media on cells viability

The two compounds used as lyoprotectants in this study were not effective in the same way on the viability of the *A. pasteurianus* cultures. In particular, all the samples of AB0220 with skim milk (20% w/w) showed high cell viability compared to the culture before the freeze-dried process (Table 1). Even after 4 days preservation of the ampoules at 37 °C, the viability was still high in the order of 10^9 cfu/mL. On the other hand, no growth was observed for all the frieze-dried cultures with mannitol.

Table 1. Assessment of viability of AB0220 strain before and after the freeze-dried

Sample	Cell count
AB0220-m before lyophilisation	2,44 x 10^9 cfu/mL
AB0220-sm before lyophilisation	1,97 x 10^9 cfu/mL
AB0220-m after lyophilisation (0 day)	No growth
AB0220-sm after lyophilisation (0 day)	1,99 x 10^9 cfu/mL
AB0220-m after 4 days at 37°C	No growth
AB0220-sm after 4 days at 37°C	1,2 x 10^9 cfu/mL

Assessment of residual moisture content

The RMC present in the freeze-dried material strongly affects viability or activity during storage. Several studies showed that for freeze-dried bacterial solutions, a high residual water content negatively affects viability during storage (Peiren et al., 2015). Residual moisture content after freeze-drying is mainly dependent on the main freeze-drying parameters such as drying temperature, pressure and time. Moreover, RMC is also dependent on the type of lyoprotectant used and even the kind of microorganism (Peiren et al., 2015). RMC was determined for all the freeze-dried AB0220 cultures. It varied with the kind of lyoprotectant used. In particular, for skim milk, it was about 1 % and a highest value of 2 % was observed with mannitol.

Effectiveness of freeze-dried AB0220 as starter culture for vinegar production

The frieze-dried culture AB0220 with skim milk was successfully applied as selected starter culture. It was able to produce about 60 g/L of AcOH after 7 days of scaling-up in wine. On the contrary, no acidity was

detected for the starter culture prepared starting from the frieze-dried culture AB0220 with mannitol (table 3).

Table 3. Detection of acidity in starter cultures obtained from the freeze-dried cultures

Starter Culture	Titratable acidity % (w/v)
AB0220-sm	5,9
AB0220-m	No acidity detected

Discussion

Vinegar technology still suffers from the lack of cost effective starter cultures due to the genetic instability of acetic acid bacteria during preservation and the susceptibility of cells to downstream processes (Shafiei et al., 2013). The viable cells, which are used as vinegar starter, must be able to tolerate acetic acid and metabolize ethanol efficiently under aggressive conditions of acetic acid fermentation (Shafiei et al., 2015).

The efficiency of *A. pasteurianus* as starter culture for vinegar depends on the cells concentration and preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in terms of viability and functional activity.

Although freeze-drying methods are successfully used for long-time bacteria preservation, they can result stressful for the cells. In fact, during the lyophilisation process the cells undergo through cold shock, which may change the physical state of the membrane lipids with consequent rupture of the cell wall. These negative effects are commonly protected by addition to the culture of some lyoprotective agents before starting the freeze-drying.

Therefore, the choice of an appropriate medium is of fundamental importance to increase the survival of organisms during and after drying, as compatible solutes are probably accumulated intracellularly. In the present study skim milk and mannitol was used as lyoprotectans and they showed a completely different effect of cells viability after freeze-drying. In particular, skim milk that provide a coating to the bacterial cells, resulted to be highly efficient. On the other hand, no viability was observed for the freeze-dried cultures with mannitol.

An organism, which survives the various steps of freezing, drying and storage, may, nevertheless, lose its viability during rehydration. In fact, rehydration is a critical step in the recovery of freeze-dried microorganisms, because cells that were subjected to sub lethal injury may not be able to

repair said damage if they are rehydrated under inappropriate conditions. In this study, one ampoule of AB0220-sm and one of AB0220-m were opened and the cell were directly rehydrated with wine in order to obtain a starter culture for vinegar production by not professional users. Also in this case only AB0220-sm was able to growth in wine and efficiently show its ability to oxidise EtOH. In conclusion, skim milk was an effective lyoprotectant able to guarantee the survival of the cells of *A. pasteurianus* strain AB0220 after freeze-drying.

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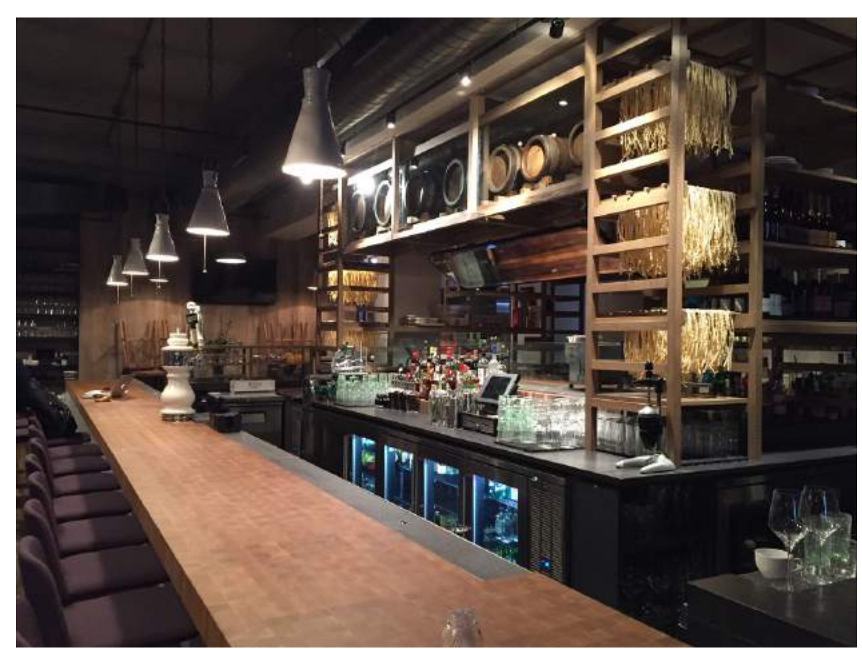
Appendix 11.

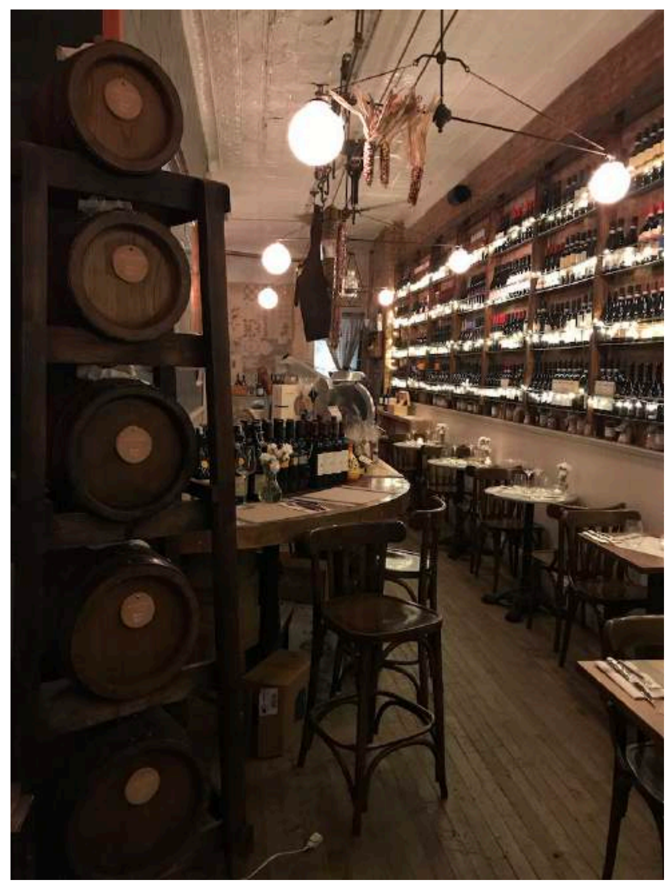
Kit for domestic production of vinegar

Barrels settled up in different location in USA for self-production of (Balsamic) vinegars.

Chicago – Monteverde Restaurant (started in 2016)



New York – Terre Restaurant (started in 2018)



New York – D'antan Restaurant (started in 2018)

Appendix 12.
Cheese Whey

Poster presented on 5th International Conference on Acetic Acid Bacteria Freising (Germany) 2018

Appendix 13.

Manuscript submitted for review

Date:	01 Oct 2019
To:	"Maria Gullo" maria.gullo@unimore.it
From:	"Biotechnology Letters (BILE)" Uthara.Udayan@springernature.com
Subject:	BILE-D-19-00819 - Submission Confirmation

Dear Dr. Gullo,

Thank you for submitting your manuscript, Genome sequencing and phylogenetic analysis of K1G4: a new Komagataeibacter strain producing bacterial cellulose from different carbon sources, to Biotechnology Letters.

The submission id is: BILE-D-19-00819
Please refer to this number in any future correspondence.

During the review process, you can keep track of the status of your manuscript by accessing the journal's website.

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1 **Genome sequencing and phylogenetic analysis of K1G4: a new *Komagataeibacter* strain**
2 **producing bacterial cellulose from different carbon sources**
3

4 **Salvatore La China[1], Andrea Bezzecchi[1], Felipe Moya[2], Giulio Petroni[3], Simona Di**
5 **Gregorio[3], Maria Gullo[1*]**
6 [1]Department of Life Sciences, University of Modena and Reggio Emilia, Reggio Emilia,
7 Italy
8 [2]Department of Agrarian and Forestry Science, Catholic University of Maule, Talca,
9 Chile
10 [3]Department of Biology, University of Pisa, Pisa, Italy
11 [*]Corresponding author at: Department of Life Sciences, University of Modena and
12 Reggio Emilia, Reggio Emilia, Italy. E-mail address: maria.gullo@unimore.it (M. Gullo).
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Abstract

Objective

The objective of this study was to evaluate the ability of K1G4 strain in producing bacterial cellulose from different carbon sources and to assess the genome sequencing with special focus on bacterial cellulose related genes.

Results

Bacterial cellulose production during 9 days of cultivation was tested in glucose, mannitol and glycerol, respectively. Differences in the bacterial cellulose kinetic formation was observed, with a final yield of 9.47 g l^{-1} in mannitol, 8.30 g l^{-1} in glycerol and 7.57 g l^{-1} in glucose, respectively. The different yield obtained using these carbon sources, makes K1G4 a versatile strain for industrial applications. The draft genome sequencing of K1G4 was produced, revealing a genome of 3.09 Mbp. Two structurally completed cellulose synthase operons and a third copy of the catalytic subunit of cellulose synthase were found. By phylogenetic analysis, using the entire rRNA operon sequence, K1G4 was found to be closely related to *Komagataeibacter xylinus* LMG 1515^{T} and *K. xylinus* K2G30.

Conclusions

K1G4 is a versatile bacterial cellulose producing strain able to produce bacterial cellulose using different carbon sources, which can be considered for scaling-up bacterial cellulose production.

Keywords bacterial cellulose, biopolymer, *Komagataeibacter xylinus,* rRNA operon.

53 **Introduction**

54 Cost-effective production of high value-added biomaterials by microbial transformations
55 is a current challenge. Nowadays there is a great interest on the discovery and
56 characterization of new biomaterials, especially focusing the environmental impact and
57 the biocompatibility features of materials (Mohite and Patil, 2014). In this scenario,
58 bacterial cellulose (BC) has a central role, as naturally produced biopolymer by bacteria.
59 BC is an ultra-fine polymer constituted by β-1,4-glucan chains. Synthetized
60 extracellularly, BC is structurally formed by nanosized fibrils, holding together by
61 hydrogen bonds (Ross et al., 1991). The polymerization process leads to the formation of
62 a coherent ultra-thin three-dimensional network of cellulose nanofibers aligned in parallel
63 to the surface of a liquid medium. This network is called pellicle and its geometry is
64 determined by intra- and intermolecular hydrogen-bonding network, hydrophobic and
65 van der Waals interactions (Gullo et al., 2018). Most of the properties that characterize
66 BC derive from the synthesis and assembling processes. Properties like purity, high
67 crystallinity and biocompatibility makes BC one of the most attractive polymers for
68 biomedical, tissue engineering, food and environmental applications. Although native BC
69 is considered adequate for different applications, actually most of researches are focused
70 on BC-based products with superior properties (Torres et al., 2019). For example, several
71 desired features are shared between scaffolds used for different tissues, such as the
72 possibility to modify the surface properties, the porosity, and the ability to design and
73 shape three-dimensional structures (Bottan et al., 2015; Robotti et al., 2018; Stasiak-
74 Różańska et al., 2018). Composite materials can be produced from BC in combination
75 with other biopolymers, such as chitosan, which is widely used in biomedical and food
76 fields. The inclusion of chitosan in the BC matrix shows an improvement of mechanical
77 properties, thermal stability, biocompatibility and antibacterial properties of the
78 composite (Kim et al., 2011; Lin et al., 2013).

79 Among different bacterial genera described as BC producers, *Komagataeibacter* genus
80 and, particularly strains of the species *K. xylinus* are foremost BC producers (Römling
81 and Galperin, 2015).
82 Within the genus *Komagataeibacter,* a high phenotypic variability of its members is
83 reported (Gullo et al., 2018). Considering BC synthesis, this variability can be observed
84 within strains recovered not only from different fermenting sources, like vinegar (Gullo
85 et al., 2016), but also from those from the same source but isolated at different time of
86 fermentation, which produces variable amount of BC (La China et al., 2018; Valera et al.,
87 2014). Moreover, differences in the structure and yield can be appreciable on the base of
88 the composition of cultivation media and the genetic organization of cellulose synthase
89 (CS) related genes.
90 To face sustainability issues, many researches are focused on the use of waste materials
91 containing high amount of sugars, like sugarcane molasses (Çakar et al., 2014; Tyagi and
92 Suresh, 2016), waste beer yeast (Lin et al., 2014), waste from fruit processing (Hungund
93 et al., 2013) and lipid fermentation wastewater (Huang et al., 2014).
94 Based on these considerations, the impact of strain selection and cultivation conditions
95 on the amount and characteristics of BC are key elements to better understand and to
96 optimize the production settings.
97 In this work, it is introduced a new *Komagataeibacter* strain isolated from Kombucha tea
98 (K2G4). The BC production ability of K1G4 was evaluated using three different media,
99 containing glucose, mannitol and glycerol, respectively. Furthermore, genome
100 sequencing was performed to understand the genetic organization of BC related genes in
101 K1G4. To infer the phylogenetic position of K1G4 the entire operon sequence, including
102 ITS regions, was analysed.
103 **Materials and methods**
104 *Bacterial strain isolation and cultivation conditions*

105 K1G4 strain was isolated from a liquid fraction of Kombucha tea as previously described
106 (Mamlouk, 2012). The strain was cultivated in aerobic conditions at 28 °C on glucose-
107 yeast extract (GY) medium: 50 g glucose l^{-1} (Merck KGaA. Darmstadt, Germany) and 10
108 g yeast extract l^{-1} (Thermo Fisher Scientific Inc.). The medium was sterilized by
109 autoclaving at 121 °C, 1 atm for 15 minutes prior the use. The strain was deposited to
110 UMCC (Unimore Microbial Culture Collection), under the collection code UMCC 2947.
111 K2G30 was handled as previously described (Gullo et al., 2019).

112 *Genome DNA extraction and sequencing*

113 Genomic DNA was extracted from a cell culture in 30 mL GY medium inoculated with
114 5% (v/v) of preinoculum and incubated at 28 °C for 5 days. Genomic DNA extraction
115 was conducted using chloroform/isoamylic alcohol method as previously described
116 (Gullo et al., 2006) and it was quantified using Qubit 2.0 (Invitrogen. Carlsbad. CA.
117 USA). A total 100 ng chromosomal DNA was used as input for the generation of Nextera
118 XT PE library. The sequencing was performed on an Illumina HiSeq X platform in a 2 X
119 150 bp run by Admera Health LCC.

120 *Genome assembly and annotation*

121 Genomic reads obtained from sequencing were quality filtered using Trimmomatic v0.36
122 tool (Bolger et al., 2014). Bases resulting with a Phred score lower than 20, were
123 discarded. Resulting high quality reads were assembled into consensus sequences using
124 SPAdes v1.10.1 (Bankevich et al., 2012), with k-mers size of 21,33,55, 77 and careful
125 option. The quality of genome assembly was evaluated using Quast v4.5 (Gurevich et al.,
126 2013). Contigs with length lower than 1 kbp were discarded. Genome annotation was
127 performed as previously described (Gullo et al., 2019). Genome raw data were deposited
128 in NCBI SRA database, under accession number SRR9326342.

129 *Phylogenetic genome reconstruction*

130 The phylogenetic reconstruction of K1G4 was carried out using the entire rRNA operon
131 (*rrn)*, including 16S, 23S, 5S rRNA genes and the internal transcribed spacers (ITS)
132 across those genes. A total of 37 genomes of *Komagataeibacter* genera were retrieved
133 from NCBI genome database, using taxonomy ID 1434011. The operon sequence dataset
134 was constructed as follow: the genomes were annotated using Barrnap v0.9 tool
135 (https://github.com/tseemann/barrnap) to predict 16S, 23S and 5S rRNA genes. The
136 coordinates obtained from rRNA gene prediction were used to retrieve the entire operon
137 sequences in the genomes. Bedtools v2.27.1 (Quinlan and Hall, 2010) was used to obtain
138 the sequences. In order to validate the datasets, a local blastn alignment was produced
139 against the 16S ribosomal RNA sequences database from NCBI using Blast v2.7.1+
140 (McGinnis and Madden, 2004). The operon sequences of type strains *Acetobacter*
141 *pasteurianus* LMG 1262 and *Acetobacter aceti* DSM 3508 were used as outgroup.
142 Retrieved sequences were aligned using Muscle v3.8.31 (Edgar, 2004). The alignment
143 was imported into MEGAX (Kumar et al., 2018) and the non-matching sequence regions
144 were clipped. From the alignment, a Maximum likelihood (ML) phylogenetic tree was
145 computed using Tamura-Nei DNA evolutionary model and a bootstrap value of 1000. A
146 discrete Gamma distribution was used to model evolutionary rate differences among sites.
147 MEGAX was also used to calculate a distance matrix among the 35 *Komagataeibacter*
148 strains, applying Tamura-nei substitution model, with a bootstrap value of 1000. In
149 addition, a Gamma distribution was applied to model the evolutionary rate differences
150 among sites. A genome-based taxonomic analysis was also performed using the average
151 nucleotide identity based on blast (ANIb) using the python pyani module (Pritchard et al.,
152 2016).
153 *Bacterial cellulose production in glucose, mannitol and glycerol media*
154 K1G4 was first cultivated in GY broth at 28 °C for 5 days under static conditions. The
155 cell were separated from cellulose film by vigorous shaking to obtain a cell suspension

156 seed culture. To test BC production, the modified Hestrin-Schramm medium (20 g yeast
157 extract l^{-1}; 5 g polypeptone l^{-1}; 2.7 g disodium phosphate anhydrous l^{-1} and 1.15 g citric
158 acid monohydrate l^{-1}) was used. The carbon source, glucose, mannitol or glycerol was
159 added at final concentration of 20 g l^{-1}. All media were sterilized by autoclaving at 121
160 °C, 1 atm for 15 min. The cultivation was conducted in 600 mL beakers with a working
161 volume of 150 mL, inoculated at 5% (v/v). Cultures were incubated at 28 °C for 9 days
162 under static conditions. Each time-point (3, 6 and 9 days) was performed in triplicate.

163 *Bacterial cellulose purification and analytical determination*

164 BC was collected at 3, 6 and 9 days of cultivation time and washed with deionized water.
165 To remove bacterial cells, BC was treated with NaOH 1 M solution and incubated at 80
166 °C for 30 minutes. Subsequently, BC was washed with deionized water until neutral pH
167 was reached. Dried BC layers were obtained by incubation at 30 °C up to a complete
168 dewatering. BC weight was expressed as previously reported (Gullo et al., 2017). For
169 each time-point, gluconic acid (GlcA), D-glucose was measured using an enzymatic kit
170 (Megazyme Ltd. Bray, Ireland), according to the manufacturer's instructions. The
171 analytic determination and pH were monitored by collect 5 ml of culture medium in each
172 time intervals. The pH was determined using an automatic titrator (TitroLine® EASY
173 SCHOTT Instruments GmbH. Mainz, Germany), equipped with an SI Analytics electrode
174 (SI Analytics. GmbH. Mainz, Germany). The samples were obtained collecting 5 mL of
175 culture medium. All determinations were conducted in triplicate.

176 **Results and discussion**

177 *Genome statistics and cellulose synthase operon description of K1G4*

178 K1G4 was previously isolated from the liquid fraction of Kombucha tea at time 0 of a
179 fermentation trial conducted by back-sloping procedure (Mamlouk, 2012). The
180 assembled genome of K1G4 covers 3.09 Mbp and consists of 169 contigs greater than 1
181 kbp, with an average in depth of 269 X (Fig. 1). A total of 2896 genes were predicted, of

182 which 2842 are protein coding genes and 51 are tRNAs. One copy of rRNA operon was
183 predicted, containing a single copy of 16S, 23S and 5S rRNA, respectively. According to
184 the KO assignment and KEGG pathway mapping, 1531 (53.9%) could be classified into
185 226 pathways. The metabolic pathways consist of the most abundant gene set (454;
186 15.97% of total CDS) in which the metabolic pathway related to BC production can be
187 found (KEGG:00500). According to COG classification, the total 2399 coding regions
188 can be assigned to 20 categories, which are described in Table 1. Genome properties and
189 statistics are summarized in Table 2.

190 Genetically, the bacteria able to produce BC are characterized by differences of CS
191 related genes organization and subunits composition. Two types of operons were
192 described in *Komagataeibacter* genus (Römling and Galperin, 2015). The first type
193 contains four genes that encode the key subunits of CS complex (*bcsA*, *bcsB*, *bcsC* and
194 *bcsD*). In addition, three genes were described in the first operon type, two of which are
195 located upstream (*cmcAx and ccpAx*) and one downstream (*bglAx*), the four *bcs* genes
196 (Matsutani et al., 2015). The second operon type contains a total of four genes: *bcsAB,*
197 *bcsX, bcsY* and *bcsC* (Römling and Galperin, 2015). It has been hypothesized that the
198 strains that possess the second operon type are able to produce an acetylated form of BC
199 due to a high similarity of BcsY with transacylase (Umeda et al., 1999). Some differences
200 in terms of operon copy number and presence/absence of the second operon type among
201 *Komagataeibacter* species were previously described (Ryngajłło et al., 2018).

202 The number of *bcs* operons spans from 1 to 3 among *Komagataeibacter* genus. Only two
203 *K. xylinus* strains were described to possess a fourth copy of *bcsAB* gene (Gullo et al.,
204 2019; Liu et al., 2018). K1G4 genome analysis revealed the presence of the two *bcs*
205 operons types structurally completed (Fig. 2). Furthermore, a third copy of *bcsAB* has
206 been found which can be correlated with the relatively high yield of BC produced.

207 Table 1

208 Table 2

209 *Phylogenetic analysis of K1G4 strain*

210 In the last decades, *Komagataeibacter* genus has suffered many phylogenetic changes. In
211 2012 the genus was reclassified as *Komagataeibacter gen. nov.,* previously part of the
212 genus *Gluconacetobacter* (Yamada et al., 2012). The phylogenetic analysis based on 16S
213 rRNA sequencing cannot provide a high resolution power (Ludwig, 2007), considering
214 that within *Komagataeibacter* genus, strains share a nucleotide 16S rRNA identity
215 percentage higher than 98.1% (Cleenwerck et al., 2010).
216 In this study, to assess the phylogenetic position of K1G4, the rRNA operon (*rrn*)
217 including 16S, 23S, 5S rRNA genes and ITS regions was analysed.
218 Prokaryotes can contain a variable number of *rrn* copies per genome (from 1 to 17 copies)
219 (Schmidt, 2011). In the present study, we first retrieved the number of *rrn* copies of 37
220 *Komagataeibacter* genomes. For most of them one copy of *rrn* has been found. For six
221 genomes, a variable *rrn* copy number has been detected (from 5 to 8) (Table 3). To
222 establish the similarity percentage of the *rrn* sequences, for each genome, a multiple
223 alignment of *rrn* copy was performed and a distance matrix was calculated. The average
224 distance values for each genome copy (data not shown) is 0, demonstrating that the
225 nucleotide sequences of the different *rrn* operons, eventually present in a single genome,
226 are identical.
227 The phylogenetic tree based on 37 rRNA operon sequences (Fig. 3) depicts diversification
228 of *Komagataeibacter* strains in two large groups, which were supported by high bootstrap
229 value (bootstrap value 100%). K1G4 has been found closely related to *K. xylinus* strain
230 K2G30 and the type strain *K. xylinus* LMG 1515^T. The phylogenetic position of K1G4 is
231 well supported by the bootstrap values, ranging from 87% with the type strain *K. xylinus*
232 LMG 1515^T and 99% with *K. xylinus* K2G30. The distance values of K1G4 and *K. xylinus*
233 K2G30 and K1G4 and *K. xylinus* LMG 1515^T are respectively 0 and 0.0007 (Table S1)

234 that means a sequence percentage similarity of 100% and 99.93%. The total average of
235 dissimilarities among all *Komagataeibacter* strains considered in this study is 0.01117,
236 which means an average sequence similarity of 98,821%. Although the majority of *K.*
237 *xylinus* strains are located in the second group, some of them clustered so far from the
238 type strain *K. xylinus* LMG 1515^T. Considering the cluster of *K. xylinus* E25, *K. xylinus*
239 E26 and *K. xylinus* BCRC 12334, the distant matrix values range from 0.0045 to 0.0054
240 that means an identity percentage from 99,46% to 99,55%. Concerning to *K. europaeus*
241 strains, which are the closest relative to the cluster of *K. xylinus* E25, E26 and BCRC
242 12334, the average values of distance ranged from 0.0016 to 0.0022 (identity percentage
243 comprises between 99.78% and 99.84%). A similar scenario can be observed from distant
244 matrix considering the strain *K. xylinus* CGMCC 2955, that is closely related to *K.*
245 *rhaeticus* cluster, and *K. xylinus* NBRC 13693, clustering with *K. oboediens* group. To
246 infer the phylogenetic relationship of these bacterial species where a clear-cut have not
247 yet been found, DNA-DNA similarity could be a suitable tool (Cleenwerck et al., 2010;
248 Lisdiyanti et al., 2006; Sievers et al., 1992). In this study ANIb analysis, was used as a
249 digital DNA-DNA hybridization tool. The results from the pairwise matrix (Table S2)
250 were consistent with the phylogenetic tree. As shown in ANIb heatmap, similar
251 clusterization of rRNA operon-based tree can be observed (Fig. 4). K1G4 shows a
252 genome sequence similarity of 99.93% with *K. xylinus* K2G30. This sequence similarity
253 is more than the recommended threshold (94%)(Umeda et al., 1999), allowing us to
254 classify K1G4 and *K. xylinus* K2G30 within the same species. About the ANIb value in
255 the pairwise comparison of K1G4 and *K. xylinus* LMG 1515^T, the similarity percentage
256 is 93.34%, just under the recommended threshold. A possible explanation of this result
257 could be due to the different isolation matrices of K1G4 and K. *xylinus* LMG 1515^T. *K.*
258 *xylinus* K1G4 and K2G30 were isolated from Kombucha tea, whereas *K. xylinus* LMG
259 1515^T was isolated from mountain ash berries. Based on NCBI taxonomy website and

260 data retrieved from different microbial culture collection website, the clusters showed in
261 the phylogenetic tree (Fig. 3) seem to follow the selective pressure due to acetic acid
262 content of the isolation matrices, except for the strains *K. kakiaceti* JCM 25156, *K.*
263 *saccharivorans* CV1 and *K. intermedius* TF2. Based on the acetic acid content, the
264 different strains can be divided into three major groups: the *K. intermedius* group, well
265 known to be isolated from vinegar with high acidity; the *K. hansenii* group, including
266 *Komagataeibacter* strains isolated from matrices with lower acetic acid content; *K.*
267 *rhaeticus* cluster, including *K. medellinensis* strain and *K. xylinus* CGMCC 2955. From
268 this point of view, the group of the *K. xylinus* strains (E25, E26 and BCRC 12334) was
269 probably mis-assigned, given the great correlation with *K. europaeus* strains and the
270 matrices condition from which were isolated. Nevertheless, it must be considered that a
271 reasonable intraspecies diversity may occur, probably due to the great variability of the
272 environmental conditions from which *Komagataeibacter* strains were isolated.
273 Table 3
274 *Cellulose production using different carbon sources*
275 The ability of K1G4 to synthetize BC was evaluated in modified HS medium using three
276 different carbon sources: mannitol, glucose and glycerol, respectively. K1G4 was able to
277 grow on all three tested media. The best carbon source in our experiment was mannitol,
278 in which a total of 9.47 g l^{-1} of BC was produced in 9 days. Considering the production
279 trend in mannitol medium, after only 3 days of cultivation, the amount of BC was
280 3.40±0.06 g l^{-1}. An appreciable increase of the BC amount has been observed at 6 days
281 (9.09±0.12 g l^{-1}). From day 6 to day 9, BC production in mannitol was low compared to
282 that produced from day 3 to day 6 (only 0.38 g l^{-1}) (Fig. 5). The trends of BC production
283 in glucose and glycerol are very similar. At the first time-point (day 3), BC production is
284 roughly near to 0, 0.192±0.014 g l^{-1} in glucose medium and 0.047±0.005 in glycerol
285 medium. From day 3 to day 6, BC amount reaches 3.99±0.05 g l^{-1} (glucose medium) and

286 3.89±0.08 l^{-1} (glycerol medium). Unlike mannitol, at the final time-point (day 9), in
287 glucose and glycerol, the amount of BC continues to increase considerably (7.57±0.08 g
288 l^{-1} and 8.30±0.08 l^{-1} respectively). The trend of BC production in mannitol can be
289 explained considering its metabolic pathway. As previously described by Oikawa and co-
290 workers, a mannitol dehydrogenase (EC 1.1.1.67) having a high affinity for the substrate
291 was found in *K. xylinus* KU-1. The mannitol dehydrogenase converts mannitol in D-
292 fructose, that can be easily used as substrate for BC production (Oikawa et al., 1997). In
293 this study, we found one copy of mannitol dehydrogenase (97% of similarity) in K1G4
294 genome based on Refseq database (Refseq id WP_082770678.1).
295 When glucose is used as a single carbon source, the multiple pathways in which it is
296 involved must be considered. The pathway of BC production starts from the
297 phosphorylation of glucose to glucose-6-phosphate (G6P) by the activity of hexokinase
298 enzyme. The G6P can be addressed into different pathways, based on the needs of the
299 cell. A reaction that carries the G6P in the BC pathways, is the interconversion of G6P to
300 glucose-1-phosphate by phosphoglucomutase. AAB are also able to produce GlcA by the
301 activity of glucose dehydrogenase , which converts the G6P into glucono- δ-lactone, an
302 intermediate of GlcA pathway, by the glucose dehydrogenase (GDH) (AMEYAMA et
303 al., 1981). From this point of view, it is possible to assume that the conversion of glucose
304 into BC or in to GlcA depends on the enzymatic kinetic features of the
305 phosphoglucomutase and the GDH, such as the substrate affinity. When glucose is
306 oxidized to gluconic derivatives, a reduction of pH values is observed (Lu et al., 2015).
307 In this study, in glucose medium (at day 9), a high amount of GlcA (11.98 g l^{-1}) was
308 detected, which was responsible for pH reduction to 3.85 and a lower BC yield compared
309 to that obtained in mannitol and glycerol media, respectively (Table 4). Knocking out of
310 GDH seems to minimize this phenomenon, avoiding the acidification of the medium and
311 increasing the BC yield (Kuo et al., 2015).

312 Considering glycerol medium, in a similar way of GDH, the activity of the membrane-
313 bound polyol dehydrogenase (PQQ-GLDH) (EC 1.1.5.2) can affect BC yield. PQQ-
314 GLDH can convert glycerol in dihydroxyacetone, reducing the carbon source availability
315 for BC production (Matsushita et al., 2003; Stasiak and Blazejak, 2009).
316 High variability in terms of BC yield was observed using different *Komagataeibacter*
317 species, like *K. hansenii* and *K. xylinus* (Gullo et al., 2018). Furthermore, intraspecies
318 variability is also reported (Gullo et al., 2017). For example, Singhsa and co-workers
319 tested four strains of *K. xylinus* species, obtaining different amount of BC (Singhsa et al.,
320 2018). Besides, in our previous work, the differences that occur among strains isolated
321 from the same matrix (Kombucha tea), after the same cultivation time, was observed. In
322 particular, in static regime of cultivation, the cellulose layer formed by strains of the *K.*
323 *xylinus* species can develop as a single or multilayer structure of different weight. (Gullo
324 et al., 2017). In this condition, the strain K2G30 produced a remarkable amount of BC
325 using mannitol as carbon source (23 g l^{-1} in 10 days) (Gullo et al., 2019).
326 Moreover, to explain the differences in BC production among *Komagataeibacter* strains,
327 we should consider also the CS operon's copy number. A higher copy number of the
328 catalytic core of CS (*bcsA; bcsB* or BcsAB) can be correlated to a higher BC production
329 (Florea et al., 2016). K1G4 possesses a total of three copies of the catalytic subunits, while
330 *K. xylinus* K2G30 isolated from the same source was reported to possess four copies of
331 the catalytic subunits (Cannon and Anderson, 1991).
332 Also, it should be paid attention the media composition. The effect of an additional
333 carbon source contributes to the maintenance of the basal metabolism of the bacterial cell.
334 The medium used in this study is a modified HS medium, which contains citric acid a
335 secondary carbon source. Citric acid can be used by bacterial cell to produce ATP via
336 Krebs cycle (Cannon and Anderson, 1991). In this way, the secondary carbon source is

337 used by cell to maintain the basal metabolism; meanwhile the primary carbon sources
338 (glucose, mannitol or glycerol) can be addressed for BC production.
339 Table 4

340 **Conclusions**

341 In this study to the aim to provide a strategy for selecting strains producing BC, an
342 approach based on metabolic, genetic and phylogenetic tools, was applied to K1G4, an
343 acetic acid bacterium previously isolated from Kombucha tea. The results demonstrated
344 that K1G4 belongs to *K. xylinus* species. K1G4 produces different BC yield when
345 growing in mannitol, glucose and glycerol media, respectively. Since, other than the high
346 yield, the production speed, is a key parameter, especially when BC is required in
347 composite materials, the versatility of K1G4, suggests the possibility to exploit it by
348 using different carbon sources according to the specific industrial use.
349 From the genomic and metabolic data, interesting inputs for the selection of strains and
350 the optimization of BC production arise.
351 Considering the phylogenetic analysis within *Komagataeibacter* genus, an alternative
352 approach has been proposed to the use of 16S rRNA, which provided a higher resolution
353 power. Using the *rrn*, small differences of sequence similarity allow to discriminate
354 strains of the *K. xylinus* species. This is of particular relevance considering that within
355 *Komagataeibacter* genus, the similarity average of taxonomic tag sequences like 16S
356 rRNA is about 98.1%.

357 **Competing interests statement**

358 The authors declare no existence of competing interests.

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361 (http://www.umcc.unimore.it/) culture collection.

362

363 **Figures and Tables**

Category	Functional group	Percentage (%)
C	Energy production and conversion	5.80
D	Cell cycle control, cell division, chromosome partitioning	0.82
E	Amino acid transport and metabolism	6.76
F	Nucleotide transport and metabolism	2.72
G	Carbohydrate transport and metabolism	5.04
H	Coenzyme transport and metabolism	3.83
I	Lipid transport and metabolism	2.38
J	Translation, ribosomal structure and biogenesis	4.97
K	Transcription	4.31
L	Replication, recombination and repair	5.49
M	Cell wall/membrane/envelope biogenesis	5.42
N	Cell motility	0.14
O	Posttranslational modification, protein turnover, chaperones	3.62
P	Inorganic ion transport and metabolism	5.11
Q	Secondary metabolites biosynthesis, transport and catabolism	1.03
S	Function unknown	20.68
T	Signal transduction mechanisms	2.45
U	Intracellular trafficking, secretion, and vesicular transport	2.17
V	Defense mechanisms	1.21

364

365 Table 1. COG categories retrieved in the K1G4 genome. The percentages reported are based on the total
366 coding regions predicted.

Genome properties	Value
Genome size	3.09 Mbp
Contigs	169
Coverage	269 X
Largest contigs	138 Kbp
G+C content	63.67 %
N50	49.9 Kbp
L50	17
Total genes	2896
of which CDS	2842
tRNA	51
Proteins with COG function	82.94%
Proteins with Kegg function	53.9%
Proteins with Pfam	80.73%

367

368 Table **2.** K1G4 genome assembly and annotation statistics.

369

370

371

Organism	*rrn* copies	Base pairs (bp)	NCBI Assembly ID	Isolation source
A. aceti DSM 3508	1	5168	GCA_004341595.1	Vinegar
A. pasteurianus LMG 1262	1	5171	GCA_000285275.1	Beer
K. cocois WE7	1	5151	GCA_003311635.1	Coconut milk
K. europaeus CECT 8546	1	5208	GCA_001273645.1	Grape vinegar
K. europaeus LMG 18494	8	5207	GCA_000227545.1	Vinegar
K. europaeus NBRC 3261	1	5207	GCA_000964485.1	Vinegar
K. europaeus SRCM101446	5	5207	GCA_002173515.1	Vinegar
K. hansenii AY201	1	5165	GCA_001645805.1	-
K. hansenii HUM-1	1	5165	GCA_001938835.1	Hummingbird feeder
K. hansenii JCM 7643	1	5165	GCA_000964405.1	-

K. hansenii LMG 23726	1	5165	GCA_001938755.1	Kombucha tea statico
K. hansenii NQ5	1	5165	GCA_001645815.1	Sugar mill
K. hansenii SC-3B	1	5165	GCA_001938745.1	Kombucha tea
K. intermedius TF2	1	5180	GCA_000964425.1	Kombucha tea
K. kakiaceti JCM 25156	1	5183	GCA_000613305.1	Kaki vinegar
K. maltaceti LMG 1529	1	5145	GCA_003206475.1	Malt vinegar
K. medellinensis NBRC 3288	5	5217	GCA_000182745.1	Vinegar
K. nataicola RZS01	5	5212	GCA_002009295.1	Rotten apples
K. oboediens 174Bp2	10	5177	GCA_000227565.1	Vinegar
K. oboediens LMG _18849	1	5177	GCA_003207815.1	Vinegar
K. pomaceti AV446	1	5147	GCA_003206055.1	Vinegar
K. pomaceti T5K1	1	5147	GCA_003207955.1	Vinegar
K. rhaeticus AF1	1	5206	GCA_000700985.1	Kombucha tea
K. rhaeticus iGEM	1	5206	GCA_900086575.1	Kombucha tea
K. saccharivorans CV1	5	5201	GCA_003546645.1	Vinegar
K. saccharivorans LMG 1582	1	5202	GCA_003207825.1	Beet juice
K. saccharivorans SRCM101450	1	5202	GCA_002878245.1	-
K. sucrofermentans LMG 18788	1	5250	GCA_003207865.1	Black cherry
K. swingsii LMG 22125	1	5241	GCA_003207895.1	Apple juice
K. xylinus BCRC 12334	1	5240	GCA_003416835.1	Vinegar
K. xylinus CGMCC 2955	1	5206	GCA_002762195.1	Vinegar
K. xylinus E25	1	5240	GCA_000550765.1	-
K. xylinus E26	1	5240	GCA_003416775.1	Vinegar
K. xylinus LMG 1515	1	5226	GCA_003207915.1	Mountains ash berries
K1G4 (This study)	1	5226		Kombucha tea
K. xylinus K2G30	1	5226	GCA_004302915.1	Kombucha tea
K. xylinus NBRC 13693	1	5177	GCA_000964505.1	-

372 **(-) Data not available**

373 Table 3. *rrn* copy number among *strains considered in this study and isolation source.*

374

	Glucose		Mannitol		Glycerol	
Time (days)	**GlcA (g l^{-1})**	**pH**	**GlcA (g l^{-1})**	**pH**	**GlcA (g l^{-1})**	**pH**
3	3.64 ± 0.6	4.79 ± 0.07	1.74 ± 0.37	5.42 ± 0.02	1.18 ± 0.19	5.33 ± 0.06
6	10.02 ± 0.58	3.87 ± 0.05	1.72 ± 0.04	5.51 ± 0.01	2.02 ± 0.18	5.52 ± 0.04
9	11.98 ± 1.48	3.85 ± 0.01	1.89 ± 021	5.5 ± 0.01	2.08 ± 0.08	5.53 ± 0.03

375

376 Table 4. Correlation between GlcA and pH during the time frame of BC production. The data were reported
377 as the average among three replicates and the standard error was included.

378

379

380 **Fig. 1.** K1G4 genome representation. From outside to inside: coverage (blue); gc_skew
381 (black positive changes and red negative changes); GC content in percentage (turquoise);
382 positive coding DNA regions (red); negative coding DNA regions (black).

383

384 **Fig. 2.** *Bcs* operons retrieved in K1G4 genome. *BcsI* is the type one of *bcs* operon,
385 containing the full set of genes codifying CS. *BcsII* is the second type of operon,
386 containing the genes codifying the CS catalytic core (*bcsAB)* and the genes encode for
387 BcsC subunit. The role of BcsX remain unclear, whereas *bcsY* gene encode a protein
388 probably responsible of the acetylated form of BC.

389

390 **Fig. 3.** Phylogenetic tree based on *rrn* operon sequences. The strains clusters were
391 highlighted based on the acidity of the different isolation matrices. (*) Different acidity
392 degree (green/high or blue/low). (?) Data not retrieved.

393

394 **Fig. 4.** ANIb values of 37 *Komagataeibacter* strains pairwise comparisons. Values are
395 represented in the central bi-color gradient heatmap (red gradients ≥ 93%; white = 90%;
396 blue gradients ≥ 90%).
397
398 **Fig. 5.** BC yields obtained during 9 days of cultivation using different carbon sources. In
399 mannitol medium, the BC production trends is very different than the glucose and
400 glycerol medium, reaching the high amount of BC during the time-point 6 days. On the
401 contrary, in glucose and glycerol media, the BC production trends is very similar, with a
402 high production rate between day 6 and 9.
403
404 Table S1. Distant matrix percentage values of pairwise comparisons for the 37
405 *Komagataeibacter* strains.
406
407 Table S2. ANIb pairwise similarity matrix.
408 **Compliance with Ethical Standards**
409 **Conflict of Interest** The authors declare that they have no conflict of interest.
410 **Ethical statement approval** This article does not contain any studies with human
411 participants or animals performed by any of the authors.
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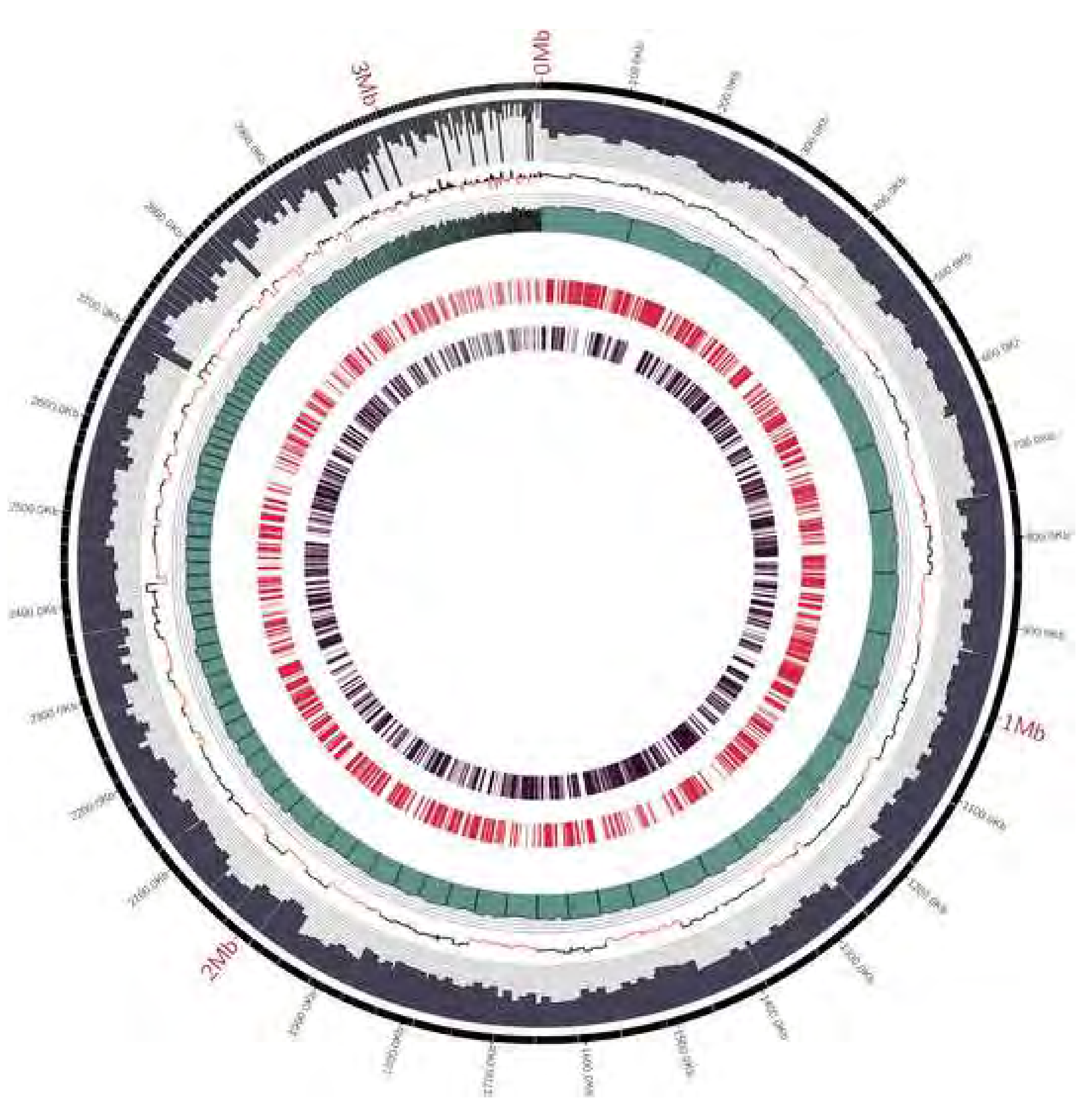

Figure
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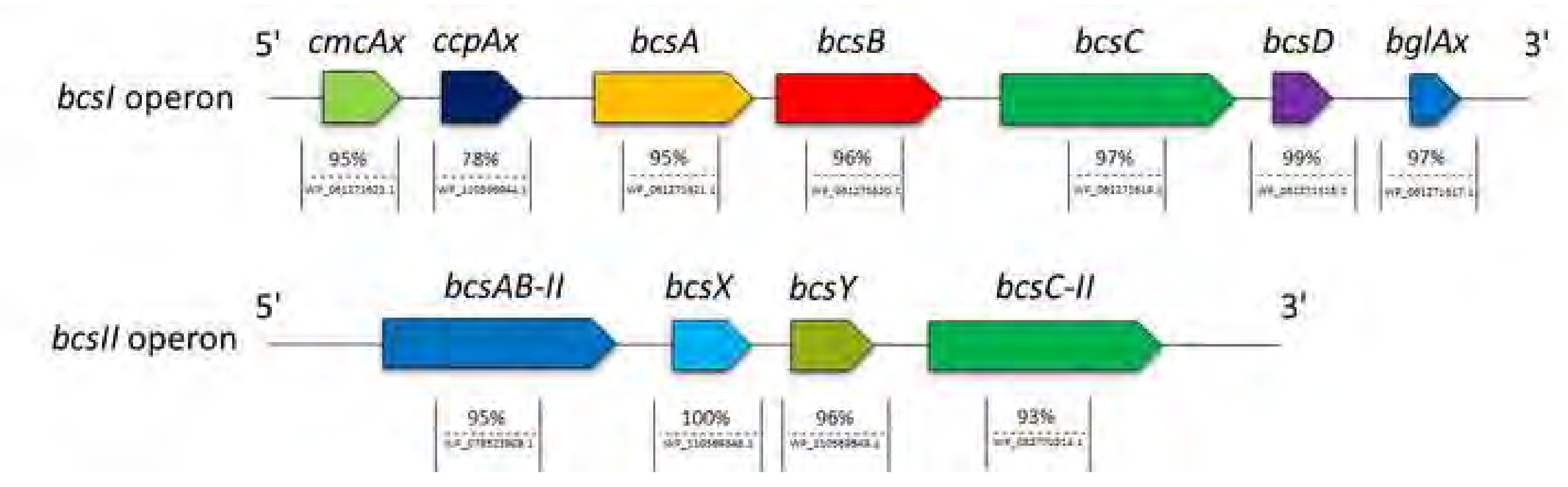

Figure

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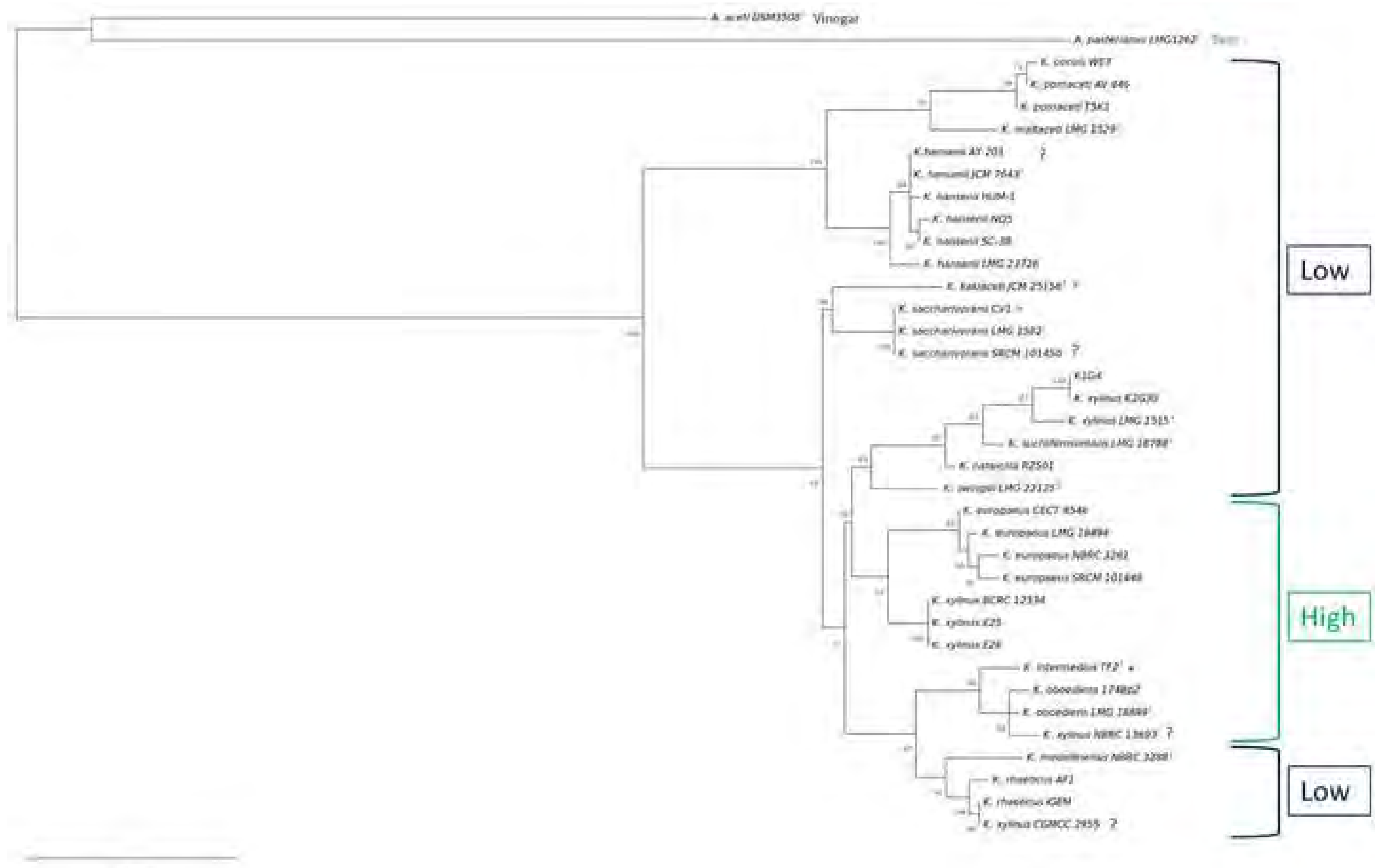

Figure

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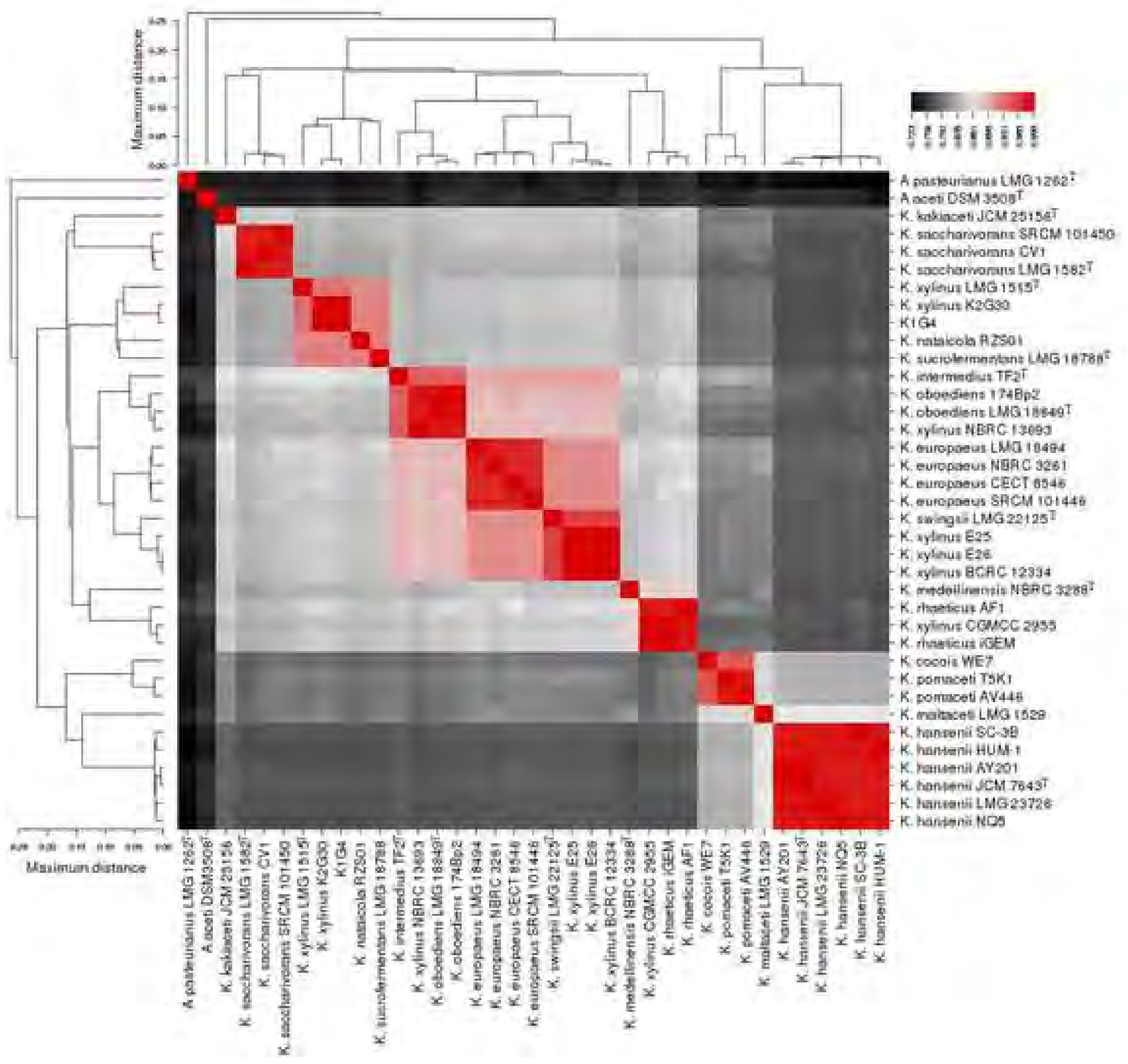

Figure

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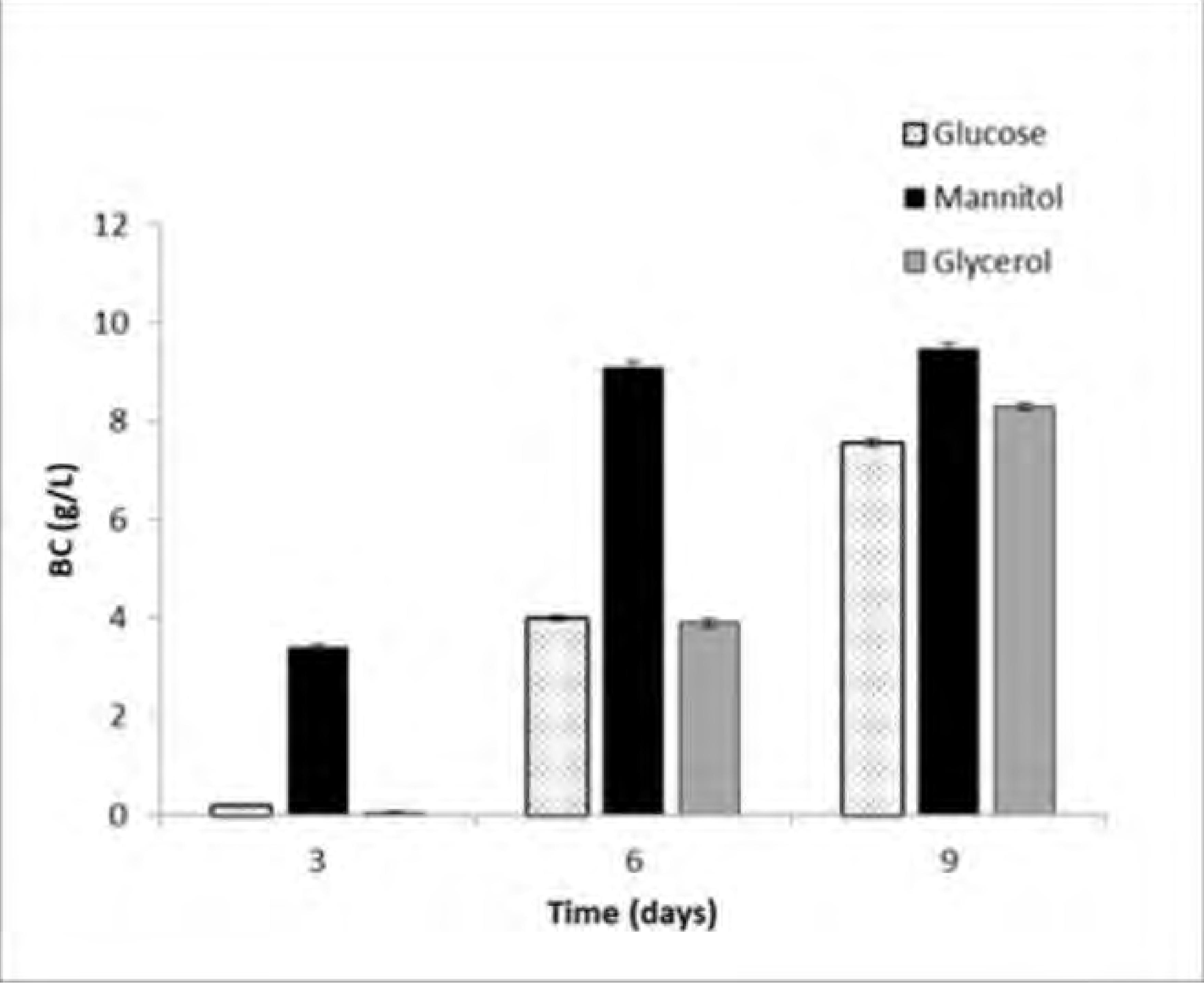

Supplementary Table S1

Click here to access/download;Table;TableS1.xls

	K1G4	K. xylinus K2G30	K. cocois WE7
K1G4			
K. xylinus K2G30	0.0000000000		
K. cocois WE7	0.0160868040	0.0160868040	
K. europaeus CECT 8546	0.0058800688	0.0058800688	0.0117909903
K. europaeus LMG 18494	0.0061569899	0.0061569899	0.0121081653
K. europaeus NBRC 3261	0.0061569688	0.0061569688	0.0127488299
K. europaeus SRCM101446	0.0064357813	0.0064357813	0.0124274774
K. hansenii ATCC 53582	0.0154002439	0.0154002439	0.0056051659
K.hansenii AY201	0.0147235969	0.0147235969	0.0056051547
K. hansenii HUM-1	0.0150606937	0.0150606937	0.0058802040
K. hansenii JCM7643	0.0147235969	0.0147235969	0.0056051547
K. hansenii LMG 23726	0.0150604595	0.0150604595	0.0058801650
K. hansenii NQ5	0.0154002439	0.0154002439	0.0056051659
K. hansenii SC-3B	0.0150607654	0.0150607654	0.0053319556
K. intermedius TF2	0.0050605234	0.0050605234	0.0127491252
K. kakiaceti JCM25156	0.0078583641	0.0078583641	0.0133985733
K. maltaceti LMG 1529	0.0154006637	0.0154006637	0.0029543141
K. medellinensis NBRC 3288	0.0045232553	0.0045232553	0.0140570422
K. nataicola RZS01	0.0029542675	0.0029542675	0.0140570351
K. oboediens 174Bp2	0.0058801006	0.0058801006	0.0137266465
K. oboediens LMG 18849	0.0056050323	0.0056050323	0.0133984169
K. pomaceti AV446	0.0157427612	0.0157427612	0.0002372013
K. pomaceti T5K1	0.0160869402	0.0160869402	0.0004759995
K. rhaeticus AF1	0.0045232544	0.0045232544	0.0121082733
K. rhaeticus iGEM	0.0045232591	0.0045232591	0.0121083044
K. saccharivorans CV1	0.0056050592	0.0056050592	0.0121082561
K. saccharivorans LMG 1582	0.0056050592	0.0056050592	0.0121082561
K. saccharivorans SRCM101450	0.0056050592	0.0056050592	0.0121082561
K. sucrofermentans LMG 18788	0.0026988729	0.0026988729	0.0143895291
K. swingsii LMG 22125	0.0045232415	0.0045232415	0.0133984764
K. xylinus ATCC 53582	0.0154002439	0.0154002439	0.0056051659
K. xylinus BCRC 12334	0.0056050674	0.0056050674	0.0108526138
K. xylinus CGMCC 2955	0.0045232591	0.0045232591	0.0121083044
K. xylinus E25	0.0056050674	0.0056050674	0.0108526138
K. xylinus E26	0.0056050674	0.0056050674	0.0108526138
K. xylinus LMG 1515	0.0014473671	0.0014473671	0.0154006227
K. xylinus NBRC 13693	0.0061569953	0.0061569953	0.0127489408

K. europaeus C	K. europaeus L	K. europaeus N	K. europaeus S	K. hansenii ATC	K.hansenii AY2
0.0002372009					
0.0007164015	0.0009584243				
0.0004759978	0.0007164021	0.0007164018			
0.0099325894	0.0102370665	0.0108521166	0.0105435978		
0.0099325568	0.0102370319	0.0108520782	0.0105435610	0.0004759979	
0.0102369753	0.0105435247	0.0111627559	0.0108521415	0.0007164021	0.0002372009
0.0099325568	0.0102370319	0.0108520782	0.0105435610	0.0004759979	0.0000000000
0.0102368628	0.0105434053	0.0111626232	0.0108520151	0.0016943165	0.0012020756
0.0099325894	0.0102370665	0.0108521166	0.0105435978	0.0000000000	0.0004759979
0.0102370097	0.0105435610	0.0111627962	0.0108521801	0.0002372009	0.0002372009
0.0042573458	0.0045232780	0.0050605238	0.0042573562	0.0121079483	0.0121079001
0.0047909785	0.0050605221	0.0056050609	0.0053318864	0.0121078618	0.0121078132
0.0099327719	0.0102372604	0.0108523328	0.0099328262	0.0045233023	0.0050605595
0.0042573364	0.0045232673	0.0050605107	0.0047909941	0.0133980981	0.0127485444
0.0042573355	0.0045232663	0.0045232547	0.0047909930	0.0133980923	0.0127485386
0.0039931955	0.0042573384	0.0047909707	0.0045232650	0.0124271071	0.0124270564
0.0037308234	0.0039931901	0.0045232348	0.0042573281	0.0121077348	0.0121076871
0.0114760693	0.0117910956	0.0124274262	0.0121082451	0.0053319719	0.0053319618
0.0117910661	0.0121082451	0.0127489178	0.0117911422	0.0056051839	0.0056051726
0.0032113842	0.0034702409	0.0039931966	0.0037308449	0.0108522677	0.0108522289
0.0037308402	0.0039932093	0.0045232594	0.0042573500	0.0114758300	0.0108522539
0.0037308355	0.0039932040	0.0045232526	0.0042573438	0.0108522539	0.0102370984
0.0037308355	0.0039932040	0.0045232526	0.0042573438	0.0108522539	0.0102370984
0.0037308355	0.0039932040	0.0045232526	0.0042573438	0.0108522539	0.0102370984
0.0039931901	0.0042573322	0.0042573220	0.0045232580	0.0130721435	0.0124270063
0.0032113776	0.0034702332	0.0039931864	0.0037308361	0.0127484960	0.0121077348
0.0099325894	0.0102370665	0.0108521166	0.0105435978	0.0000000000	0.0004759979
0.0016943212	0.0019429355	0.0024452003	0.0021932262	0.0096303291	0.0096302980
0.0037308402	0.0039932093	0.0045232594	0.0042573500	0.0114758300	0.0108522539
0.0016943212	0.0019429355	0.0024452003	0.0021932262	0.0096303291	0.0096302980
0.0016943212	0.0019429355	0.0024452003	0.0021932262	0.0096303291	0.0096302980
0.0042572900	0.0045232150	0.0045232036	0.0047909355	0.0140560371	0.0133976140
0.0037308281	0.0039931955	0.0045232418	0.0042573343	0.0108521976	0.0114756809

K. hansenii HUI	K. hansenii JCN	K. hansenii LM(	K. hansenii NQ!	K. hansenii SC-	K. intermedius ⁻
0.0002372009					
0.0009584234	0.0012020756				
0.0007164021	0.0004759979	0.0016943165			
0.0004759978	0.0002372009	0.0014473695	0.0002372009		
0.0124271480	0.0121079001	0.0124269838	0.0121079483	0.0117908137	
0.0124270564	0.0121078132	0.0124268922	0.0121078618	0.0117907313	0.0050605564
0.0053319179	0.0050605595	0.0053318858	0.0045233023	0.0047910252	0.0114761157
0.0130721601	0.0127485444	0.0130719799	0.0133980981	0.0130722146	0.0039932279
0.0130721538	0.0127485386	0.0130719736	0.0133980923	0.0130722089	0.0039932272
0.0127484763	0.0124270564	0.0127483042	0.0124271071	0.0121078132	0.0016943251
0.0124269243	0.0121076871	0.0124267614	0.0121077348	0.0117906110	0.0014473745
0.0056051616	0.0053319618	0.0056051260	0.0053319719	0.0050605992	0.0124277076
0.0058802236	0.0056051726	0.0058801845	0.0056051839	0.0053319719	0.0121084334
0.0111629149	0.0108522289	0.0111627817	0.0108522677	0.0111629556	0.0029542845
0.0111629414	0.0108522539	0.0111628082	0.0114758300	0.0111629819	0.0029542865
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0.0105435953	0.0102370984	0.0105434764	0.0108522539	0.0105436311	0.0039932254
0.0105435953	0.0102370984	0.0105434764	0.0108522539	0.0105436311	0.0039932254
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0.0124269742	0.0121077348	0.0124268106	0.0127484960	0.0124270246	0.0050605419
0.0007164021	0.0004759979	0.0016943165	0.0000000000	0.0002372009	0.0121079483
0.0099326664	0.0096302980	0.0099325596	0.0096303291	0.0099326992	0.0045232971
0.0111629414	0.0108522539	0.0111628082	0.0114758300	0.0111629819	0.0029542865
0.0099326664	0.0096302980	0.0099325596	0.0096303291	0.0099326992	0.0045232971
0.0099326664	0.0096302980	0.0099325596	0.0096303291	0.0099326992	0.0045232971
0.0137256436	0.0133976140	0.0137254481	0.0140560371	0.0137257033	0.0067164574
0.0117906110	0.0114756809	0.0117904631	0.0108521976	0.0111628814	0.0014473753

K. kakiaceti JCI	K. maltaceti LM	K. medellinensis	K. nataicola RZ	K. oboediens 17	K. oboediens LI
0.0133984080					
0.0056051040	0.0133984233				
0.0050605419	0.0133984169	0.0039932188			
0.0053318968	0.0124273885	0.0042573533	0.0047910014		
0.0050605170	0.0121080013	0.0039932032	0.0045232622	0.0007164022	
0.0130727596	0.0026989191	0.0137267647	0.0137267576	0.0133986104	0.0130726103
0.0133986706	0.0024452349	0.0140571484	0.0140571413	0.0137267482	0.0133985135
0.0056051027	0.0114760439	0.0024452078	0.0045232822	0.0032113940	0.0029542709
0.0056051097	0.0121081680	0.0024452092	0.0045232868	0.0032113963	0.0029542729
0.0034702481	0.0121081198	0.0039932172	0.0029542785	0.0047909988	0.0045232600
0.0034702481	0.0121081198	0.0039932172	0.0029542785	0.0047909988	0.0045232600
0.0034702481	0.0121081198	0.0039932172	0.0029542785	0.0047909988	0.0045232600
0.0064358350	0.0130724528	0.0053318902	0.0016943224	0.0056050793	0.0053318613
0.0045232679	0.0127487908	0.0039932086	0.0029542739	0.0058801254	0.0056050547
0.0121078618	0.0045233023	0.0133980981	0.0133980923	0.0124271071	0.0121077348
0.0039932196	0.0096305019	0.0034702528	0.0034702523	0.0053319031	0.0050605227
0.0056051097	0.0121081680	0.0024452092	0.0045232868	0.0032113963	0.0029542729
0.0039932196	0.0096305019	0.0034702528	0.0034702523	0.0053319031	0.0050605227
0.0039932196	0.0096305019	0.0034702528	0.0034702523	0.0053319031	0.0050605227
0.0072835004	0.0140563888	0.0050604807	0.0029542573	0.0069989981	0.0067163896
0.0056050831	0.0108524141	0.0045232701	0.0050605274	0.0012020787	0.0009584248

K. pomaceti AV	K. pomaceti T5I	K. rhaeticus AF	K. rhaeticus iGE	K. saccharivora	K. saccharivora
0.0002372013					
0.0117911983	0.0121083532				
0.0117912279	0.0121083842	0.0004759982			
0.0117911820	0.0121083356	0.0034702490	0.0034702518		
0.0117911820	0.0121083356	0.0034702490	0.0034702518	0.0000000000	
0.0117911820	0.0121083356	0.0034702490	0.0034702518	0.0000000000	0.0000000000
0.0140570008	0.0143896399	0.0053318889	0.0047909988	0.0042573443	0.0042573443
0.0130726670	0.0133985733	0.0050605274	0.0050605331	0.0034702412	0.0034702412
0.0053319719	0.0056051839	0.0108522677	0.0114758300	0.0108522539	0.0108522539
0.0105440322	0.0108526788	0.0034702523	0.0039932242	0.0024452078	0.0024452078
0.0117912279	0.0121083842	0.0004759982	0.0000000000	0.0034702518	0.0034702518
0.0105440322	0.0108526788	0.0034702523	0.0039932242	0.0024452078	0.0024452078
0.0105440322	0.0108526788	0.0034702523	0.0039932242	0.0024452078	0.0024452078
0.0150612477	0.0154007476	0.0056050266	0.0056050336	0.0050604769	0.0050604769
0.0124275316	0.0127490289	0.0029542739	0.0034702454	0.0045232669	0.0045232669

K. saccharivora	K. sucroferment	K. swingsii LMG	K. xylinus ATCC	K. xylinus BCR(	K. xylinus CGM
0.0042573443					
0.0034702412	0.0042573348				
0.0108522539	0.0130721435	0.0127484960			
0.0024452078	0.0042573491	0.0024452046	0.0096303291		
0.0034702518	0.0047909988	0.0050605331	0.0114758300	0.0039932242	
0.0024452078	0.0042573491	0.0024452046	0.0096303291	0.0000000000	0.0039932242
0.0024452078	0.0042573491	0.0024452046	0.0096303291	0.0000000000	0.0039932242
0.0050604769	0.0021932133	0.0034702122	0.0140560371	0.0045232340	0.0056050336
0.0045232669	0.0058801137	0.0061570223	0.0108521976	0.0045232725	0.0034702454

K. xylinus E25	K. xylinus E26	K. xylinus LMG 1515	K. xylinus NBRC 13693
0.0000000000			
0.0045232340	0.0045232340		
0.0045232725	0.0045232725	0.0072834712	

Divergence between Sequences
from between sequences are shown.

of evolutionary distance between
and Tamura K. (2018). MEGA X:
ensure the correctness of the caption, the caption text

Supplementary Table S2

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	K. xylinus E26	K. pomacet	K. xylinus B	K. hansenii	K. maltacet
K. xylinus E26	1.0	0.8039384	0.9993164	0.7771116	0.7997817
K. pomaceti T5K1	0.807077866061294	1.0	0.8074000	0.8313351	0.8527281
K. xylinus BCRC 12334	0.9992901000571756	0.8023882	1.0	0.7762465	0.7984379
K. hansenii AY201	0.7774649254790632	0.8320366	0.7775775	1.0	0.8628820
K. maltaceti LMG 1529	0.8006755646100114	0.8523200	0.8005017	0.8631343	1.0
K. hansenii SC-3B	0.7788620147255689	0.8283881	0.7784195	0.9848345	0.8594974
K. hansenii LMG 23726	0.7768234089392928	0.8277053	0.7767120	0.9862088	0.8609453
K. saccharivorans CV1	0.835478072677093	0.7874005	0.8343507	0.7738186	0.7913549
K. xylinus K2G30	0.8464603131991052	0.7953798	0.8466616	0.7713327	0.7980054
K. hansenii HUM-1	0.7760345027247957	0.8287600	0.7757180	0.9878654	0.8601991
K. nataicola RZS01	0.8412917921960074	0.7916268	0.8409707	0.7712288	0.7925135
K. xylinus ATCC 53582	0.7763086926286509	0.8276511	0.7763801	0.9875606	0.8619455
K. europaeus LMG 18494	0.9166916186012978	0.8141828	0.9159748	0.7828513	0.8186448
K. europaeus NBRC 3261	0.914595275735294	0.8101705	0.9136606	0.7801240	0.8211991
K. xylinus CGMCC 2955	0.8462044470152706	0.7822099	0.8459779	0.7752154	0.7852677
K. intermedius TF2	0.90264322515213	0.8318934	0.9020683	0.7818051	0.8201582
K. swingsii LMG 22125	0.9614519931623932	0.8071720	0.9611058	0.7766970	0.8021096
K. xylinus LMG 1515	0.8444910925844669	0.7942340	0.8446660	0.7711246	0.7933768
K. cocois WE7	0.7916470030211481	0.9477599	0.7913936	0.8324275	0.8491808
K. saccharivorans LMG 1582	0.8321838997078871	0.7802334	0.8315391	0.7754336	0.7826093
K. saccharivorans SRCM101450	0.842672205289108	0.8024178	0.8423672	0.7732407	0.8042686
K. hansenii JCM 7643	0.7798518582375479	0.8300210	0.7793265	0.9982847	0.8602400
K. medellinensis NBRC 3288	0.838305720202485	0.7889291	0.8372557	0.7696300	0.8085083
K. hansenii ATCC 53582	0.7772128639455782	0.8290511	0.7776065	0.9877671	0.8625224
K. oboediens LMG 18849	0.8884709517871987	0.7991720	0.8880272	0.7759655	0.7943349
K. sucrofermentans LMG 18788	0.8416913777161349	0.7930371	0.8419504	0.7720158	0.7979007
K. kakiaceti JCM 25156	0.8439188780713955	0.7963385	0.8433077	0.7790352	0.7995194
K. xylinus NBRC 13693	0.8853235870029929	0.7881802	0.8848490	0.7736535	0.7888053
K. hansenii NQ5	0.7766250210674157	0.8281971	0.7767519	0.9878660	0.8618927
K1G4	0.8431391096532334	0.7880690	0.8424147	0.7696689	0.7929406
K. rhaeticus iGEM	0.8508530474531999	0.7932360	0.8511314	0.7738584	0.7956891
K. oboediens 174Bp2	0.8939552214714713	0.8083261	0.8935516	0.7858512	0.8057685
K. pomaceti AV446	0.7974201160092809	0.9848365	0.7975709	0.8318231	0.8503987
K. xylinus E25	0.9982361650341346	0.8054903	0.9984522	0.7768139	0.8014970
K. europaeus CECT 8546	0.917161067961165	0.8080489	0.9158571	0.7814379	0.8060142
K. europaeus SRCM101446	0.9165911720510894	0.7991764	0.9146536	0.7766729	0.8031602
K. rhaeticus AF1	0.8543476758147515	0.8048104	0.8536652	0.7770703	0.7983373

K. hansenii	K. hansenii	K. sacchari	K. xylinus K	K. hansenii	K. nataicola	K. xylinus ATCC 53	K. europaeus LM
0.7778992	0.7786660	0.8329619	0.8461238	0.7766041	0.8382770	0.7763282951653	0.916533842201
0.8291519	0.8281021	0.7924691	0.8018126	0.8284148	0.7915528	0.8279321679316	0.80638542046
0.7772655	0.7770933	0.8321193	0.8466628	0.7756287	0.8380279	0.7747266320293	0.91508030528
0.9845799	0.9875042	0.7745064	0.7739418	0.9880351	0.7725196	0.9873125618682	0.778046604152
0.8598406	0.8613948	0.7943460	0.8029663	0.8594225	0.7903321	0.8612600661430	0.805626855142
1.0	0.9870885	0.7750544	0.7751250	0.9881648	0.7748089	0.9872179251269	0.77855983322
0.9862251	1.0	0.7722246	0.7714884	0.9877655	0.7716462	0.9881368695380	0.77579298969
0.7760693	0.7743269	1.0	0.8284268	0.7699256	0.8256470	0.7699983089637	0.83443544943
0.7727424	0.7705973	0.8257958	1.0	0.7691431	0.9179064	0.7698533784655	0.84155310490
0.9885103	0.9885127	0.7691954	0.7707276	1.0	0.7711725	0.9887336667738	0.77425120792
0.7823735	0.7719213	0.8283976	0.9210862	0.7711404	1.0	0.7710515117801	0.838931689277
0.9877653	0.9894323	0.7695371	0.7714892	0.9892104	0.7705912	1.0	0.775206528428
0.7851643	0.7850370	0.8416211	0.8514451	0.7795938	0.8442053	0.7812676521204	1.0
0.7861593	0.7810550	0.8380492	0.8479386	0.7773843	0.8424527	0.7792423143564	0.981376562595
0.7778668	0.7745343	0.8291197	0.8409919	0.7713414	0.8337616	0.7715633051398	0.84854096153
0.7887496	0.7848142	0.8551372	0.8624057	0.7762723	0.8513983	0.7765685508982	0.898229300064
0.7784571	0.7780129	0.8334887	0.8451280	0.7755544	0.8394137	0.7761745414294	0.91678147879
0.7771154	0.7703410	0.8254563	0.9317553	0.7682088	0.9141765	0.7693005277044	0.840304529841
0.8279634	0.8276594	0.7879519	0.7897159	0.8272129	0.7837843	0.8281436476190	0.799300606394
0.7741515	0.7729956	0.9841099	0.8219522	0.7711785	0.8198024	0.7719608841259	0.82975547738
0.7754013	0.7735995	0.9834279	0.8365201	0.7709217	0.8282890	0.7718478192534	0.84655956539
0.9836148	0.9867771	0.7754360	0.7779563	0.9874996	0.7756733	0.9874255106319	0.78041615384
0.7722647	0.7754420	0.8226438	0.8344425	0.7664746	0.8297879	0.7747576560509	0.843570699784
0.9879887	0.9895962	0.7696871	0.7724327	0.9891481	0.7714751	0.9998863684834	0.775876848325
0.7798887	0.7752482	0.8317013	0.8408423	0.7746697	0.8336275	0.7741334716732	0.88849993783
0.7722186	0.7721621	0.8244777	0.9248000	0.7693856	0.9248864	0.7710311976047	0.843929512831
0.7797237	0.7789318	0.8447787	0.8382403	0.7785169	0.8339260	0.7785099036608	0.84469501096
0.7741754	0.7746284	0.8291760	0.8348199	0.7747473	0.8308534	0.7737621948051	0.887170336577
0.9877938	0.9897211	0.7699070	0.7711462	0.9891016	0.7706028	0.9998898854386	0.77516797579
0.7724765	0.7690569	0.8223799	0.9993203	0.7684535	0.9203969	0.7678851384199	0.83887567230
0.7775631	0.7726847	0.8305987	0.8481553	0.7702160	0.8365087	0.7713293706293	0.85014991603
0.7817779	0.7793965	0.8389714	0.8486121	0.7776975	0.8369088	0.7799940586797	0.891073819494
0.8298165	0.8292077	0.7980101	0.7961084	0.8282890	0.7880488	0.8283404029643	0.802799095315
0.7780214	0.7784254	0.8343464	0.8495884	0.7761521	0.8399788	0.7753276139240	0.91489243963
0.7801185	0.7784044	0.8365004	0.8513541	0.7761101	0.8394255	0.7778502194357	0.977261544611
0.7803356	0.7777097	0.8395426	0.8442575	0.7748665	0.8351959	0.7758760566037	0.98038763564
0.7856998	0.7748210	0.8339676	0.8528208	0.7717954	0.8384559	0.7719165245478	0.849228567901

K. europae	K. xylinus C	K. intermed	K. swingsii	K. xylinus L	K. cocois W	K. sacchariv	K. sacchariv	K. hansenii
0.9154032	0.8431006	0.8923875	0.9622485	0.8429719	0.7932491	0.8311981	0.8389800	0.7779085
0.8018667	0.7828532	0.8008670	0.8100248	0.7980931	0.9476005	0.7818154	0.8009984	0.8285435
0.9141593	0.8426321	0.8911905	0.9614224	0.8433814	0.7918990	0.8301443	0.8383384	0.7768600
0.7748203	0.7754511	0.7789083	0.7766426	0.7723850	0.8315757	0.7743099	0.7752913	0.9998618
0.8113422	0.7860903	0.8004357	0.8034656	0.7955084	0.8502748	0.7847430	0.8022142	0.8596291
0.7751577	0.7758783	0.7796489	0.7783795	0.7752112	0.8284826	0.7720823	0.7751653	0.9872505
0.7741344	0.7718401	0.7765847	0.7756643	0.7705814	0.8274314	0.7715106	0.7729143	0.9886515
0.8345370	0.8285720	0.8342814	0.8352320	0.8246522	0.7879566	0.9839998	0.9849866	0.7732503
0.8394168	0.8345123	0.8398691	0.8437785	0.9331953	0.7870609	0.8209767	0.8311512	0.7717400
0.7738053	0.7713915	0.7750395	0.7751584	0.7693274	0.8284981	0.7703332	0.7703055	0.9894893
0.8359954	0.8324027	0.8354558	0.8418197	0.9182000	0.7887953	0.8235685	0.8278490	0.7716209
0.7743933	0.7704671	0.7757823	0.7750368	0.7695401	0.8282571	0.7707490	0.7717980	0.9897644
0.9805384	0.8475670	0.8929093	0.9176884	0.8482146	0.8142334	0.8319954	0.8546970	0.7831531
1.0	0.8409415	0.8932600	0.9155802	0.8452900	0.8041540	0.8323432	0.8453983	0.7807981
0.8397044	1.0	0.8396513	0.8456792	0.8384073	0.7811615	0.8261672	0.8295786	0.7748543
0.8997467	0.8402050	1.0	0.9044347	0.8604502	0.8172521	0.8453090	0.8586318	0.7818762
0.9140995	0.8426205	0.8918195	1.0	0.8436492	0.7951956	0.8307483	0.8399038	0.7772225
0.8370665	0.8351555	0.8368070	0.8442302	1.0	0.7849678	0.8222881	0.8273843	0.7717482
0.7931621	0.7800962	0.7882333	0.7947917	0.7842001	1.0	0.7787082	0.7936052	0.8298728
0.8291529	0.8247717	0.8341801	0.8309742	0.8208131	0.7795032	1.0	0.9839007	0.7754342
0.8431974	0.8265879	0.8397189	0.8429571	0.8309472	0.7982454	0.9803117	1.0	0.7741627
0.7776709	0.7776150	0.7817198	0.7787638	0.7762228	0.8295945	0.7764105	0.7749681	1.0
0.8467099	0.8704142	0.8357296	0.8392360	0.8295588	0.7942268	0.8172150	0.8295388	0.7701636
0.7756163	0.7713191	0.7760493	0.7763873	0.7708288	0.8295472	0.7714577	0.7723454	0.9899301
0.8867236	0.8366514	0.9422511	0.8872970	0.8394424	0.7917138	0.8299241	0.8339047	0.7769554
0.8380459	0.8318030	0.8374513	0.8428857	0.9204899	0.7902646	0.8194959	0.8305958	0.7724134
0.8448595	0.8353859	0.8399633	0.8433269	0.8342922	0.7975611	0.8419640	0.8486841	0.7792276
0.8875122	0.8365793	0.9445329	0.8850222	0.8317329	0.7889118	0.8278186	0.8315224	0.7742854
0.7754453	0.7705493	0.7759229	0.7756748	0.7698413	0.8277219	0.7708707	0.7724769	0.9900334
0.8363754	0.8346552	0.8354703	0.8417785	0.9334768	0.7834510	0.8194185	0.8267370	0.7701618
0.8419000	0.9965679	0.8444348	0.8494987	0.8430399	0.7880794	0.8257417	0.8344615	0.7740049
0.8880379	0.8390240	0.9434456	0.8914679	0.8438542	0.8048522	0.8335988	0.8450402	0.7853423
0.7966057	0.7826892	0.7940852	0.7994587	0.7909589	0.9467009	0.7804079	0.7968298	0.8291115
0.9137325	0.8441171	0.8912598	0.9615802	0.8448068	0.7932078	0.8329081	0.8410757	0.7779695
0.9783451	0.8392284	0.8919606	0.9164328	0.8465074	0.8046470	0.8310034	0.8436900	0.7810240
0.9802908	0.8406832	0.8905003	0.9164553	0.8406824	0.7957054	0.8304403	0.8423384	0.7771884
0.8446875	0.9841633	0.8474533	0.8526327	0.8482619	0.7935915	0.8293190	0.8360964	0.7768005

K. medellin	K. hansenii	K. oboedie	K. sucrofer	K. kakiaceti	K. xylinus N	K. hansenii	K1G4	K. rhaeticus
0.8364339	0.7773740	0.8884147	0.8410138	0.8464832	0.8844161	0.7763639	0.8410113	0.8503598
0.7940789	0.8288146	0.7958488	0.7961156	0.8090053	0.7894339	0.8279030	0.7923775	0.7985657
0.8354733	0.7760432	0.8874424	0.8408878	0.8451806	0.8833977	0.7746728	0.8410108	0.8498759
0.7687122	0.9876623	0.7754239	0.7718828	0.7833708	0.7730613	0.9873443	0.7716627	0.7760021
0.8122292	0.8617487	0.7941298	0.8010312	0.8069470	0.7866064	0.8612788	0.7937172	0.7986324
0.7715782	0.9873747	0.7763030	0.7722935	0.7821423	0.7726655	0.9871418	0.7737019	0.7786088
0.7724124	0.9882461	0.7732624	0.7717822	0.7808405	0.7717021	0.9880957	0.7698871	0.7728222
0.8216511	0.7705733	0.8323376	0.8266230	0.8485548	0.8284686	0.7702181	0.8240656	0.8321001
0.8306795	0.7706115	0.8374506	0.9236823	0.8398177	0.8309102	0.7697822	0.9976346	0.8441429
0.7657792	0.9887918	0.7727035	0.7701472	0.7814109	0.7727388	0.9886495	0.7698886	0.7719298
0.8317422	0.7717201	0.8359853	0.9239085	0.8382400	0.8294009	0.7710999	0.9220207	0.8385047
0.7727455	0.9998980	0.7745898	0.7715628	0.7806954	0.7726376	0.9998388	0.7690007	0.7717401
0.8526263	0.7819588	0.8916313	0.8534707	0.8586167	0.8883724	0.7810356	0.8459845	0.8555869
0.8524538	0.7799882	0.8900240	0.8452279	0.8521849	0.8870081	0.7793277	0.8444018	0.8474581
0.8719279	0.7722604	0.8423749	0.8345033	0.8387166	0.8354140	0.7714422	0.8363834	0.9976233
0.8524969	0.7774810	0.9434686	0.8513726	0.8607276	0.9424282	0.7766485	0.8552819	0.8592308
0.8375217	0.7770897	0.8881620	0.8430830	0.8449392	0.8838300	0.7761998	0.8416999	0.8500483
0.8283278	0.7700677	0.8395704	0.9177018	0.8361987	0.8292657	0.7693087	0.9314451	0.8430672
0.7929019	0.8289767	0.7904544	0.7907050	0.8049264	0.7842167	0.8281272	0.7846830	0.7895666
0.8144279	0.7724608	0.8296971	0.8198023	0.8432500	0.8268022	0.7718660	0.8201945	0.8262848
0.8317063	0.7722613	0.8351965	0.8347583	0.8537882	0.8301641	0.7717669	0.8290702	0.8357001
0.7722396	0.9876550	0.7756630	0.7744915	0.7843857	0.7737420	0.9874508	0.7743746	0.7780245
1.0	0.7754389	0.8321851	0.8340542	0.8392583	0.8279017	0.7744859	0.8301221	0.8737947
0.7731006	1.0	0.7751255	0.7725108	0.7826371	0.7733271	0.9997935	0.7697795	0.7725226
0.8302913	0.7745366	1.0	0.8333016	0.8408680	0.9843089	0.7740241	0.8350396	0.8436887
0.8330462	0.7717508	0.8338169	1.0	0.8381457	0.8283086	0.7709779	0.9245267	0.8401331
0.8309540	0.7790950	0.8387796	0.8355042	1.0	0.8359416	0.7785037	0.8359503	0.8410319
0.8286784	0.7743495	0.9865108	0.8316998	0.8415131	1.0	0.7736676	0.8329605	0.8396897
0.7729300	0.9998477	0.7736836	0.7715846	0.7820052	0.7726125	1.0	0.7689743	0.7718705
0.8291247	0.7684346	0.8340374	0.9242383	0.8395085	0.8298048	0.7678468	1.0	0.8420379
0.8733523	0.7719237	0.8433525	0.8405274	0.8433434	0.8366294	0.7712692	0.8403153	1.0
0.8383626	0.7808772	0.9924882	0.8385351	0.8508587	0.9849137	0.7795842	0.8403684	0.8496544
0.7907786	0.8288073	0.7913910	0.7925120	0.8037394	0.7849596	0.8281095	0.7899807	0.7921889
0.8371509	0.7761865	0.8877014	0.8429452	0.8476350	0.8841304	0.7752793	0.8423592	0.8523503
0.8440809	0.7788997	0.8931562	0.8444139	0.8517172	0.8862976	0.7777927	0.8430827	0.8509865
0.8373050	0.7765677	0.8863641	0.8419701	0.8466100	0.8851316	0.7759114	0.8386223	0.8484888
0.8725065	0.7730213	0.8490840	0.8390847	0.8461974	0.8382393	0.7718067	0.8437234	0.9857541

K. oboedie	K. pomacet	K. xylinus E	K. europae	K. europae	K. rhaeticus AF1
0.8886260	0.7981628	0.9994728	0.9172797	0.9157161	0.8499363643961555
0.8007504	0.9845048	0.8103018	0.8079104	0.8036766	0.8035558852459016
0.8874084	0.7966677	0.9996171	0.9152625	0.9137680	0.8489733643724695
0.7777865	0.8330804	0.7769665	0.7816604	0.7777816	0.7782082749504297
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0.7762406	0.8302491	0.7781920	0.7780551	0.7801296	0.7798883678593849
0.7723747	0.8286736	0.7759560	0.7763835	0.7760337	0.772764578778135
0.8337396	0.7981834	0.8346704	0.8360524	0.8395177	0.8351458516601984
0.8407486	0.7953430	0.8457126	0.8453193	0.8419655	0.8489562362525459
0.7729979	0.8285733	0.7751309	0.7753818	0.7746746	0.7724906644518272
0.8336209	0.7911364	0.8408976	0.8416574	0.8380084	0.8420856734517106
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1.0	0.8043702	0.8923430	0.8982645	0.8876179	0.8573061205341033
0.7947753	1.0	0.7984566	0.8001494	0.8020420	0.7955626730444569
0.8885126	0.7988326	1.0	0.9154666	0.9127115	0.8514261283728537
0.8953840	0.8060006	0.9160169	1.0	0.9789620	0.8586610974201001
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0.8528041	0.7990828	0.8532847	0.8592893	0.8472819	1.0

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