Fermented probiotic beverage based on quinoa

Functionality, hygiene, and health effects

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2021

Document Version:
Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (APA):
Canaviri Paz, P. R. (2021). *Fermented probiotic beverage based on quinoa: Functionality, hygiene, and health effects*. [Doctoral Thesis (compilation), Division of Food and Pharma]. Food Technology, Lund University.

Total number of authors:
1

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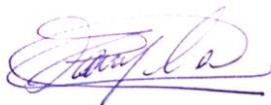| **Organization** LUND UNIVERSITY	**Document name:** Doctoral thesis
Department of Food Technology, Engineering and Nutrition, LTH	**Date of issue**
Author(s): Pamela Rosario Canaviri Paz	Sponsoring organization: Swedish International Development Cooperation Agency (SIDA)

Title and subtitle: Fermented probiotic beverage based on quinoa (Functionality, hygiene, and health effects)

Abstract

White quinoa grains were used as a source of novel beneficial microorganisms and as a matrix for development of a fermented plant-based drink. Studies on the quinoa grain microbiota were performed by applying spontaneous fermentation to allow isolation of autochthonous *Lactiplantibacillus* strains with potential to be used as starter cultures. The quinoa-based drink was fermented with the commercial probiotic strain *Lactiplantibacillus plantarum* DSM9843 (=299v), or with autochthonous *Lactiplantibacillus* strains. The efficiency of the strains as starter cultures were determined by monitoring changes in the bacteriological community during fermentation. Additionally, interactions between polyphenols and the strains during fermentation were analysed in the beverage and finally, in an attempt to categorize one of the strains as future probiotics, the modified microbiota composition in healthy volunteers was determined after consumption.

The experimental procedure was designed to characterize the grain microbiota on the surface as well as inside the grains through preparation of a liquid quinoa dough fermented spontaneously at 30 °C for 8 days. Samples were cultured and viable cells were isolated and genetically analysed applying Sanger sequencing. A consortium of potential pathogenic and beneficial bacteria co-existed and changed positively during quinoa dough fermentation, with the *Lactobacillaceae* family overtaking the niche. Isolates of autochthonous *Lactiplantibacillus* strains were further investigated and a phenotypical characterization of the enzymatic capacity of the strains on different carbohydrates and on degradation of tannins were performed. The results showed that the *L. plantarum* strains were able to ferment a large array of carbohydrates, including xylose and glycerol. Furthermore, tannase degradation was also observed for most of the tested strains.

When using starter cultures to ferment the quinoa-based beverage, the commercial strain *L. plantarum* DSM9843 proved to be a strong inhibitor of undesirable microorganisms for up to 28 days of storage time. However, viable cells of *Enterococcus* spp., remained present, questioning the efficiency of the commercial strain as starter culture and the limitations of using pH values of 4 or below as parameter of safeness. As a second approach, the quinoa grains were toasted, mixed with sterile water, and inoculated with four of the previously isolated strains: *L. plantarum* 3, *L. plantarum* 5, *L. plantarum* 9, and *L. plantarum* 10, respectively. Mapping the bacterial community by next generation sequencing (NGS) showed that Firmicutes dominated after fermentation.

Changes in the content of polyphenolic compounds were analysed using reversed-phase high-performance liquid chromatography (RP-HPLC). No significant variation was observed in the total content of polyphenols or flavonoids between beverages fermented with the different strains, but significant variations were observed for different compounds such as gallic acid, vanillic acid, syringic acid, quercetin 3O-glucoside and rutin.

Furthermore, one of the isolated strains *L. plantarum* 3, was tested as a first step to categorize the bacterium as a future probiotic strain. Healthy volunteers consumed the fermented quinoa-based beverage for 14 days. Saliva and stool samples were collected and analysed qualitatively and quantitatively by quantitative-polymerase chain reaction (q-PCR), terminal restriction fragment length polymorphism (T-RLFP) and NGS. A modified microbiota composition was found and the increase of the amount of *Lactobacillaceae* species may indicate that the bacterium did survive the transit through the gastrointestinal tract.

Key words: Quinoa grains, microbiota characterization, fermentation, food hygiene, novel *L. plantarum* strains, polyphenols, Next Generation Sequencing, High-Performance Liquid Chromatography.

Classification system and/or index terms (if any)		
Supplementary bibliographical information		**Language:** English
ISSN and key title		ISBN 978-91-7422-824-3 (print) 978-91-7422-825-0 (PDF)
Recipient's notes	**Number of pages 77**	Price
	Security classification	

Signature _(signature)_   Date 2021-08-23

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"To do a great and important work,
two things are necessary
- a definite plan,
and not quite enough time"

-Elbert Hubbard

Table of Contents

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Popular science summary

That the best things come in small packages is a well-known saying and food is no exception to this idea. Quinoa is a small grain with big value for the food industry. Quinoa is a native plant typical of the Andean Altiplano in South America. The seeds are compact with a high content of proteins, essential amino acids, and carbohydrates along with minerals, vitamins, and polyphenols with antioxidative properties. Additionally, quinoa seeds are gluten free. Due to those extraordinary properties, quinoa seeds are included in a variety of staple foods.

A revolution in the food industry boosted by the so-called plant-based diet is ongoing, and it demands food alternatives based on plants and functional foods (def., as "foods with extraordinary benefits for health"). Therefore, using quinoa as a "main ingredient" instead of as a "supplementary ingredient" was the background of this thesis, in which a quinoa-based drink was developed. To optimize the health beneficial effects of quinoa, probiotic bacteria were added to the quinoa-based drink. Probiotics are good bacteria with health benefits. As a result, a functional beverage was developed to comply with the plant-based diet demands.

Considering this, the following activities were planned: 1) Developing a quinoa drink by adapting effective food safety and quality control systems to ensure a proper hygiene; 2) Searching for a novel, beneficial and safe bacterium able to multiply in the beverage and inhibit potential pathogenic bacteria; 3) Establishing the most suitable conditions for fermentation of the quinoa-based drink; 4) Analysing the changes in polyphenolic compounds before and after fermentation, and finally; 5) Evaluating the possibility to modify the microbiota composition in healthy volunteers after consumption of the fermented quinoa-based drink.

After preparation of the quinoa-based drink, a high concentration of potentially harmful bacteria was detected. Heat treatment was therefore not sufficient to ensure a consumable product. The drink was then fermented with *Lactiplantibacillus plantarum* 299v, a commercial probiotic strain. *L. plantarum* 299v proved to be a strong inhibitor of undesirable microorganisms, although fermentation for 2 days was not enough to achieve adequate food safety (Paper I).

After characterizing the microbiota of quinoa seeds, pathogenic bacteria like *Enterobacteriaceae* were found in high amounts, but beneficial bacteria belonging to the *Lactobacillaceae* and *Leuconostocaceae* families, such as *Lactobacillus* species and *Pediococcus* species were also discovered. The finding of the

Lactobacillaceae family naturally occurring on quinoa seeds opened the possibility to search for suitable bacterial species applicable for fermentation processes. Through culturing, viable cells were obtained and isolates of the *Lactiplantibacillus* spp. were identified and their characteristics were described. An important finding was the ability of the bacterial species to metabolize specific carbohydrates and to handle oxidative environments (Paper II).

Four of the isolated *L. plantarum* strains were further evaluated for hygienic aspects and for their potential to be used as starter cultures. The results showed dominance and survivability of the tested strains and an efficiency to secure the safety of the beverages. Since the fermentation process involves several chemical reactions, small molecules like polyphenolic compounds may also vary in concentrations. In the present study, it was demonstrated that several compounds were affected to different extents by the fermentation process (Paper III).

In the last study, a fermented beverage based on quinoa was developed using one of the isolated strains. The fermented drink was consumed by healthy volunteers to determine if the bacterium possessed potential as probiotics, which is requiring survivability thought the gastrointestinal tract. Saliva and stool samples were analysed before and after the intake of the drink, and the results showed a modified microbiota composition (Paper IV).

The work of this thesis points out knowledge gaps that were tried to be filled. Further improvements of, for example, flavour and smell of the product are needed before it can be acceptable for consumption. Deeper investigation of the health beneficial effects and the functionality of *Lactiplantibacillus* spp. isolates are also required.

Populärvetenskaplig sammanfattning

Att de bästa sakerna kommer i små paket är ett välkänt uttryck och mat är inget undantag. Quinoafröet är ett litet frö med stort värde för matindustrin. Quinoa härstammar från den Andiska högplatån i Sydamerika. Quinoafrön är kompakta med högt innehåll av proteiner, essentiella aminosyror, kolhydrater samt mineraler, vitaminer, och ämnen så som polyfenoler med anitoxidativa egenskaper. Utöver detta så är även quinoa glutenfritt. Tack vare dessa extraordinära egenskaper är quinoa inkluderat i en mängd olika basfödor.

Det pågår en revolution inom matindustrin med grund i det som kallas för en växtbaserad kost, vilket medför krav på alternativa produkter baserade på växtermaterial och med ett mervärde (d.v.s., livsmedel som kan förbättra hälsan). Med vetenskapen som grund utvecklades i denna avhandling en quinoabaserad dryck, med fokus på användning av quinoa som en huvudingrediens istället för som en tillsats. För att optimera de nyttiga egenskaperna hos quinoa tillsattes probiotiska bakterier till drycken. Probiotika är goda bakterier som ger hälsofördelar. Resultatet av arbetet är en funktionell dryck som möter efterfrågan på en mer växtbaserad kost.

För arbetet planerades följande aktiviteter: 1) Utveckla en quinoabaserad dryck med fokus på livsmedelssäkerhet och kvalitetskontroll; 2) Finna en hälsofrämjande bakterie med förmåga att föröka sig i drycken samt förhindra tillväxt av potentiellt sjukdomsframkallande bakterier; 3) Fastställa de mest gynnsamma betingelserna för fermentering av drycken; 4) Studera förändringar i polyfenolsammansättningen hos quinoa efter fermentering, och slutligen; 5) Analysera möjligheten att påverka tarmflorans sammansättning i friska, frivilliga personer efter konsumtion av drycken.

Efter framställning av den quinoabaserade drycken upptäcktes en hög koncentration av potentiellt sjukdomsframkallande bakterier. Behandling med hög värme var inte tillräckligt för framtagning av en säker dryck. Drycken fermenterades därför med *Lactiplantibacillus plantarum* 299v, en kommersiellt tillgänglig probiotisk bakterie. *L. plantarum* 299v visade sig vara en stark hämmare av de oönskade mikroorganismerna, men trots att fermenteringen pågick i två dagar så var det inte tillräckligt för att säkerställa en god livsmedelshygien.

Vid karakteriseringen av de naturligt förekommande mikroorganismerna i quinoafrön upptäcktes sjukdomsframkallande bakterier så som *Enterobacteriaceae* i stora mängder, men även hälsofrämjande bakterier som tillhörde

Lactobacillaceae- och *Leuconostocaceae* familjerna, t.ex. *Lactobacillus* spp., och *Pediococcus* spp. Upptäckten av naturligt förekommande bakterier tillhörande *Lactobacillaceae* familjen i quinoafrön öppnade upp möjligheten att utföra en djupare undersökning med målet att finna en lämplig bakteriestam för fermentering. Isolat av *Lactiplantibacillus* spp., identifierades och beskrevs fenotypiskt. En viktig upptäckt var bakteriestammarnas förmåga att metabolisera specifika kolhydrater och deras förmåga att hantera oxidativa förhållanden.

Fyra av de *L. plantarum* stammar som isolerades från quinoa utvärderades med hänsyn till hygienaspekter, för att utröna om de var lämpliga som starterkulturer. Resultatet visade på dominans och överlevnadsförmåga hos de testade stammarna och en effektiv förmåga att säkerställa god hygien hos dryckerna. Eftersom fermenteringsprocessen involverar flera kemiska reaktioner kan små molekyler så som polyfenolföreningar också variera i koncentration. I den här avhandlingen påvisas det att flera av dessa föreningar förändras på olika sätt under fermenteringen.

I avhandlingens sista studie utvecklades en fermenterad dryck baserad på quinoa med en av de isolerade bakterierna med probiotisk förmåga. Den fermenterade drycken konsumerades av friska frivilliga personer och saliv- och avföringsprover analyserades före och efter intag. Resultaten visade en modifierad bakterieflora i tarmen.

Denna avhandling har identifierat kunskapsbrister och presenterar försök att åtgärda dessa. Framtida förbättringar av bland annat smak och doft krävs för att drycken ska bli acceptabel för konsumtion. Det behövs också djupare undersökningar av *Lactiplantibacillus* stammarnas hälsofrämjande egenskaper och funktionalitet.

List of publications

This thesis is based on research work scientifically disclosed in the following publications and manuscripts listed below in order by Roman numerals:

I. **Canaviri-Paz**, **P**., Janny, R. J., Håkansson, Å. (2020). Safeguarding of quinoa beverage production by fermentation with *Lactobacillus plantarum* DSM9843. *Int J Food Microbiol. 2020;324:108630.* https://doi.org/10.1016/j.ijfoodmicro.2020.108630.

II. **Canaviri-Paz**, **P**., Oscarsson, E., Håkansson, Å. (2021). Autochthonous microorganisms of white quinoa grains with special attention to novel functional properties of lactobacilli strains. *J Funct Foods. 2021;84: 104586.* https://doi.org/10.1016/j.jff.2021.104586.

III. **Canaviri-Paz**, **P**., Fredom-Gondo, T., Kjellström, A., Mandoga, T., Sithole, J., Oscarsson, E., Sandahl, M., Håkansson, Å. (2021). Synergy between autochthonous *Lactiplantibacillus plantarum* spp. and polyphenolic compounds during quinoa-based drink fermentation. (*Manuscript*)

IV. **Canaviri-Paz**, P., Oscarsson, E., Kjellström, A., Olsson, H., Jois, C., Håkansson, Å. (2021). Effects on microbiota composition after consumption of quinoa beverage fermented by a novel xylose-metabolizing *L. plantarum* strain. (*Submitted manuscript*)

Published articles not related to the thesis

- Burri, S., Granheimer, K., Rémy, M., Tannira, V., So, Y., Rumpunen, K., Tornberg, E., **Canaviri-Paz**, **P**., Uhlig, E., Oscarsson, E., Rohrstock, A-M., Rahman, M., Håkansson, Å. (2021). Processed meat products with added plant antioxidants affect the microbiota and immune response in C57BL/6JRj mice with cyclically induced chronic inflammation. *Biomed Pharmacother. 2021;135*:111133.
  https://doi.org/10.1016/j.biopha.2020.111133.

Contribution to the papers

The author´s contribution to the published paper and manuscripts listed above were:

I. I planned, designed, and performed the formulation of the quinoa-based beverage, the fermentation process, and the microbiological analyses as well as the pH and acidity measurements. The master student Rownoke Jannat Janny was under my supervision developing different pasteurization trials. I analysed the data and wrote the draft of the manuscript. I made improvements of the manuscript along with Åsa Håkansson.

II. I planned, designed, and performed the experiments during fermentation, identification of the isolated microorganisms and discrimination between *Lactiplantibacillus* spp., at genotypical and phenotypical level. I analysed the data and wrote the draft of the manuscript. I made corrections and improvements along with the co-authors.

III. I planned, designed, performed, and developed the formulation of the fermented quinoa-based drink. The students Tawanda Mandoga and Jaison Sithole performed their diploma work under my supervision. I made the microbiological analysis by NGS in collaboration with the co-authors Anna Kjellström, Elin Oscarsson and Åsa Håkansson. I planned, designed, and performed the analytical analyses by HPLC together with the co-authors Fredom Thamani Gondo and Margareta Sandahl. I analysed the data and wrote the draft of the manuscript. I performed the corrections and improvements of the manuscript in collaboration with all co-authors.

IV. I planned, designed, and performed the fermentation of the quinoa-based drink. The student Chandana Jois performed her diploma work under my supervision. I planned, designed, performed, and enrolled volunteers in the human trial. The student Hanna Olsson conducted her diploma work under my supervision. I planned and performed the experimental work along with the co-authors. I analysed the data and wrote the manuscript draft. I revised, corrected, and improved the manuscript in collaboration with Elin Oscarsson and Åsa Håkansson.

Contributions to seminars and conferences

Canaviri-Paz, **P**. Bacterias probioticas (*Oral presentation*). Seminario "Biotecnologia y Bioprocesos: Alternativas tecnologicas Aplicadas en Bolivia. July 6-7th, 2017. La Paz-Bolivia.

Canaviri-Paz, **P**., Mandoga, T., Jois, C., Ahrné, S., Håkansson, Å., Development of fermented quinoa milk by different *Lactobacillus* strains. (*Poster presentation*) 33rd EFFoST International Conference 2019. November 12-14th, 2019, Rotterdam-Netherlands.

Abbreviations

ANOVA	Analysis of variance
ASV	Amplicon sequence variant
CFU	Colony forming units
DF	Dilution factor
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
GRAS	Generally regarded as safe
LAB	Lactic acid bacteria
La	Lactic acid
MEA	Malt extract agar
MRS	de Man, Rogosa and Sharpe
NGS	Next generation sequencing
PCR	Polymerase chain reaction
q-PCR	Quantitative polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase high-performance liquid chromatography
SBS	Sequencing by synthesis
T-RFLP	Terminal restriction fragment length polymorphism
TSA	Tryptic soy agar
VRBD	Violet red bile dextrose agar

1.Introduction

A race towards developing commercial products with added health benefits based on empirical and scientific knowledge has begun. A deeper understanding of the human body and the role of the gastrointestinal tract as a link between diet and health was the starting point and the background to how functional foods emerged, with the purpose to meet specific requirements and prevent diseases (Shandilya, 2017).

To keep the gut microbiota in balance, consumption of probiotics and prebiotics is conducive (Almeida et al., 2012). Probiotic microorganisms can also be used as starter cultures and combined with prebiotic substances to optimize product quality and to simplify the industrial fermentation process, where time is a pivotal factor. However, the microbiological characteristics and quality of organisms and substrate used is of high importance for product safety, and the believe in fermentation as a safe process is sometimes overestimated, posing a risk to consumer health (Motawee & Neveen, 2016).

Commercially available probiotics are applied to induce fermentation in order to shorten production time and because these microorganisms are considered safe. The alternative fermentation process, spontaneous fermentation, depends on the naturally occurring microorganisms in the raw material and require a longer period of time for a successful fermentation. However, spontaneous fermentation provides the advantage of discovering novel microorganisms that can be used as starter cultures and as probiotics (Vera-Pingitore et al., 2016). Prebiotics are generally non-digestible parts of plant materials, such as fibres (Jeske et al., 2018) and because of being a natural source, plant-based fermented products have also become a strategic niche of research to include phytochemical compounds such as polyphenols in the diet and to isolate novel beneficial bacterial strains.

Polyphenolic compounds are plant secondary metabolites synthesized as a response to the environmental and growth conditions. Polyphenolic compounds are of high interest in functional foods due to their antioxidant capacity as free oxygen radical scavengers and it has repeatedly been shown that quinoa seeds are one of the major sources of polyphenolic compounds among cereals and pseudocereals (Rocchetti et al., 2017).

Quinoa seeds were initially introduced as prebiotics in the formulation of functional foods (Bianchi et al., 2015). However, due to the extraordinary physicochemical

and phytochemical properties of quinoa, a spectrum of different quinoa-based products has emerged. Furthermore, autochthonous, novel beneficial bacteria from the *Lactiplantibacilaceae* and *Leuconostocaceae* family has been isolated from the grains and been tested as starter cultures and even as potential probiotics (Vera-Pingitore et al., 2016).

The present research work focusses on the functionality, hygiene and health effects of a fermented probiotic beverage based on quinoa. The procedure includes an effective fermentation time along with a simple formulation using quinoa seeds as matrix and *L. plantarum* strains as starter cultures, to ensure food safety of the beverage. The beverage functionality was evaluated by the content of polyphenols as well as by pH and the amount of lactic acid (La). Finally, the possibilities of the novel strain *L. plantarum* 3 to affect the microbiota was analysed in healthy volunteers after intake of the beverage, as a first step to categorize the strain as probiotic in the future.

1.1. Overview of the thesis

The present work is based on:

Hygiene and safety (Paper I)

- o Formulation of a fermented quinoa-based drink.
- o Effectiveness of a commercial starter culture in enhancing microbiological hygiene and safety.

Microbiology (Paper II)

- o Characterization of the native microbiota of quinoa grains.
- o Differentiation of autochthonous *Lactiplantibacillus plantarum* strains at genotypical and phenotypical level.

Functionality (Paper III)

- o Identification and quantification of bioactive secondary metabolites, such as polyphenols in the quinoa-based matrix after fermentation.
- o Identification and quantification of organic acids produced by enzymatic actions during fermentation.

Health effects (Paper IV)

- o *In vivo* trial of the fermented quinoa-based drink.
- o Microbiota composition after consumption of the drink.

1.2. Goals of the research

The main goal of the thesis was to improve the health benefits of the polyphenol-rich Andean quinoa grain through fermentation with potentially probiotic bacteria.

The specific goals were:

1. Formulation and optimization of a quinoa-based drink used as substrate for fermentation with *Lactiplantibacillus* strains.

2. Characterization of the naturally occurring microbiota on fermented quinoa grains, with special attention to isolation of autochthonous *Lactiplantibacillus* strains.

3. Identification of polyphenolic compounds and microbiota composition before and after fermentation, and during storage time of the fermented quinoa-based drink.

4. Evaluation of changes in microbiota composition in healthy volunteers after consumption of the quinoa-based drink fermented by a novel autochthonous *Lactiplantibacillus plantarum* strain.

2.The Golden Grain

The seeds are the most frequently consumed part of the quinoa plant, and they are used in different foods as well as for development of new food products. The functionality of quinoa has been deeply investigated from the biological, chemical, and physicochemical point of view. The strive for greener production methods, such as fermentation, and for development of healthier and more sustainable plant-based products positions quinoa as an attractive alternative. Quinoa seeds contain carbohydrates, proteins, lipids, minerals, and vitamins as the macrostructures and phytochemicals such as polyphenols, saponins, organic acids, and phytosterols, constitute the microstructures. Furthermore, in recent years special attention has been focused on the characterization of the quinoa microbiota (Franco et al., 2020; Ruiz-Rodríguez et al., 2016; Vera-Pingitore et al., 2016) finding bacteria such as *Enterococcus* spp., *Leclercia* spp., *Lactococcus* spp., *Lactobacillus* spp., and *Pediococcus* spp., among others.

2.1. The beginning

The quinoa plant, worldwide known as *Chenopodium quinoa* type species, part of the genera *Chenopodium L.*, and family *Chenopodiaceae* was classified by Carl Ludwig von Willdenow[1] in his book "Species Plantarum". Edtio Quarta. Berolini (Berlin) published in July 1798. The plant also received *Amaranthaceae* as the family name given by Antoine Laurent de Jussieu in his work "Genera Plantarum" published on August 4th, 1789[2]. Both family names were accepted and considered synonyms based on the morphological characteristics described by the mentioned authors. However, recent molecular studies of the plant DNA (Fuentes-Bazan et al., 2012; Kadereit et al., 2012; Zou et al., 2017) suggested that *Amaranthaceae* and *Chenopodiaceae* are in fact closely-related family members and graded them as sisters (Fuentes-Bazan et al., 2012; Jørgensen et al., 2014).

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[1] The family name *Chenopodiaceae* is accepted by the **International Plant Names** Index where the data is given by the authors.

[2] *Amaranthaceae* Juss., information available at the **International Plant names**. https://www.ipni.org.

The quinoa history goes back to "The Incas Empire". The Incas were an organized civilization with a territory expansion occupying part of Ecuador, Perú, Bolivia, Chile and Argentina (Bushnell et al., 2021). The variability of quinoa is exceptionally high, and 6721 accessions have been reported from which 3178 belong to the Bolivian germplasm collection INIAF - (Instituto Nacional de Innovación Agropecuaria y Forestal) (Rojas et al., 2014). The vast variety is positioning Bolivia as the number one country having the largest germplasm collection of quinoa in the world, followed by Perú (Basantes-Morales et al., 2019; Rojas et al., 2014). Therefore, the Andean highlands of Bolivia has been appointed as the quinoa centre of origin (Balize et al., 2015).

Figure 1. Quinoa plants (©Katherin-Fermandez), and Titicaca Lake (©Adilen-Fermandez) from Bolivia 2018-2019.

The quinoa plant is mainly domesticated around the Titicaca Lake at 3800 m above sea level (Pfeifer et al., 2006), widely known as the highest lake in the world (Figure 1). This position was chosen by the Incas as the optimal area to cultivate quinoa due to the environmental conditions (Jellen et al., 2015). Quinua is the proper pronunciation in Spanish, and it is used in South America, while it is Quinoa in English, and that pronunciation is used in the rest of the world. The adaptability of the quinoa plant to different ecological floors and subsequent climate changes (García-Parra et al., 2020), granted the quinoa plant as "The Golden Grain" being the most nutritional seed (Silva et al., 2020). Furthermore, in recognition of the Incas agronomic heritage domesticating the quinoa plant and, in an effort to preserve this knowledge through time, the Food and Agriculture Organization of the United Nations (FAO), appointed 2013 as the "International Quinoa Year" (FAO, 2013).

2.2. The quinoa seed

2.2.1. Macromolecules and their applications

The macromolecular structure of quinoa and the nutritional properties linked to it position quinoa as a high quality and nutritional seed (Nowak et al., 2016; Repo-Carrasco et al., 2003). Generally, carbohydrates are divided into sugars (monosaccharides and disaccharides), starch (oligosaccharides and polysaccharides) and fibres. The sugars in quinoa seeds consist of a low proportion of glucose and fructose, making the plant a good alternative for development of low glycaemic index products (Pineli et al., 2015). The higher content of D-ribose, D-galactose, maltose and D-xylose (Ogungbenle, 2003) makes it frequently used in the formulation of beverages (Deželak et al., 2014) as well as in the biorefinery industry (Paniagua Bermejo et al., 2020; Salas-Veizaga et al., 2017). Quinoa seeds are composed of more than 55 % of starch (Valcárcel-Yamani & Lannes, 2012). The physicochemical properties of quinoa starch are molecularly defined by the low content of amylose and the structural conformation of amylopectin, which consists of a high number of short chains (Inouchi et al., 1999). Thus, quinoa starch gelatinizes at temperatures between 65.8 °C to 74.9 °C, which was proven after studies of 26 different commercial quinoa seeds (Li et al., 2016). Therefore, a vat pasteurization process at 63 °C for 30 minutes, adapted from protocols used by the dairy industry (International Dairy Foods Association, n.d.) was applied in Paper I. Satisfactory results were obtained in regard to the quinoa-based beverage stability, which did not present any sedimentation or formation of clumps after pasteurization (Paper I). Quinoa seeds contain in total 10% of dietary fibres, of which 78% constitute insoluble dietary fibres composed of galacturonic acid, arabinose, xylose, glucose, galactose, and rhamnose (Lamothe et al., 2015).

The amino acids considered essential in our diet are tryptophan, valine, histidine, methionine, lysine, phenylalanine, tyrosine, threonine, isoleucine, and leucine, and it is also these amino acids that define the quality of the proteins (S. A. Valencia-Chamorro, 2003; Villa et al., 2014). Quinoa has a high protein content compared to cereals and the seeds provide all of those 10 essential amino acids, thereby presenting an excellent protein profile (Vega-Gálvez et al., 2010). In quinoa, globulins and albumins are the major protein components excluding prolamins, which is the protein responsible for causing the chronic bowel enteropathy known as celiac disease (Alvarez-Jubete et al., 2009; Håkansson et al., 2019; Valcárcel-Yamani & Lannes, 2012). Therefore, quinoa is a good alternative for individuals suffering from celiac disease or gluten allergy.

2.2.2. Micromolecules

Many micromolecules, including phytochemicals also called secondary metabolites, have been identified and isolated from quinoa (Pereira et al., 2020). The bioactivity of these micromolecules are considered beneficial to the plant, conferring protection against animals and as biological response to environmental stress (Hinojosa et al., 2018; Jacobsen et al., 2003, 2007). However, the same principle is not entirely applicable to humans, where the dose of secondary metabolites defines their physiological effects (Scalbert & Williamson, 2000; Wan et al., 2020). Quinoa grains have been studied due to their content of polyphenols. Polyphenolic compounds constitute a group of secondary metabolites of natural organic molecules with large multiples of phenol structural units, showing capacity as antioxidants (Chandrasekara & Shahidi, 2011). Saponins are another group of secondary metabolites of interest in quinoa since they are responsible for the bitter flavour. The content of saponins must be less than 0.02% of the quinoa seeds before human consumption (Guzman et al., 2013) and therefore, depending on the amount of ingested saponins, they are differentiated as nutrients or as antinutrients.

According to Hemalatha *et. al.*, the most frequently isolated secondary metabolites from quinoa seeds are gallic acid, 3,4-dihydroxybenzoic acid, vanillic acid (hydroxybenzoic acid derivatives), chlorogenic acid, p-coumaric acid, sinapic acid, ferulic acid, (hydroxy cinnamic acid derivatives), catechin, rutin, myricetin, daidzein, luteolin, quercetin, apigenin, naringenin and kaempferol (flavonoids) (Hemalatha et al., 2016), most of them also identified in Paper III. Additionally, a study by Kuljanabhagavad *et. al.*, focusing on isolation and identification of saponins, presented a total of 23 saponins from milled quinoa seeds (Kuljanabhagavad et al., 2008).

To estimate the content of polyphenolic compounds in food is difficult due to their structural diversity, but also due to loss or enrichment of compounds during processing (Scalbert & Williamson, 2000). Furthermore, the concentration of polyphenols needed to exert beneficial health effects and protection against disease development is currently not known (Bo et al., 2019). Measurements of dietary intake is complicated, especially for micronutrients and bioactive compounds, for which the intake depends on dietary habits, geographical location, and population characteristics such as age, gender, and socio-cultural factors (Bo et al., 2019). An estimation of total polyphenol intake for the overall population has recently been estimated to 900 mg per day and that concentration corresponds to what has previously been reported (Bo et al., 2019). Although the polyphenolic compounds have been identified in the food, it does not necessarily mean that they are accessible for humans.

2.3. Functional foods

In principle, all foods (from plant-, animal- or microbial origin) are functional foods because they provide nutrients, prevent diseases, and help the body to recover by providing physiological benefits (Hasler, 2002). Presently, there are no universally accepted terms to clarify "Functional food" (FAO, 2007), but based on four principles (Figure 2) the following working definition has been generated (Doyon & Labrecque, 2008):

"A functional food is, or appears similar to, a conventional food. It is part of a standard diet and is consumed on a regular basis, in normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions".
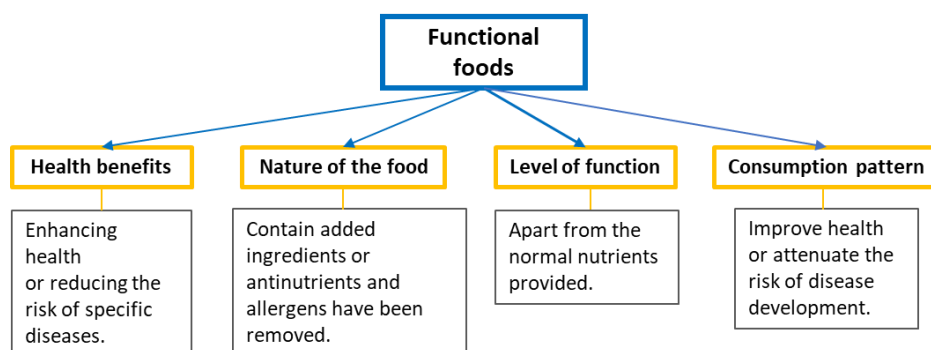
Figure 2. The four principles used to define a functional food (Doyon & Labrecque, 2008).

Due to the potential of functional foods to improve health and well-being of consumers, the global demand is continuously increasing, prompting further research and development of new products by the industry. Gastrointestinal function is one of the most promising targets for functional food science (Roberfroid, 2000). For this purpose, fermented foods have unique potentials containing microorganisms providing probiotic functions and biotransformation of the raw materials used, which in turn enhance bioavailability of nutrients, degrade antinutrients, and enrich with bioactive compounds (Tamang et al., 2016).

Except for having a history of being produced for preservation and food safety reasons, fermented beverages are easy to consume and good vehicles for transportation of accessible nutrients and bioactive compounds (Ghoshal & Kansal, 2019). To optimise the interactions between the microorganisms and the substrate constituents, one of the main steps in the design and development of functional beverages is selection of suitable ingredients. Plant-derived foods including cereals and pseudocereals are rich sources of both fibre and bioactive compounds and also

utilise abundant natural resources to ensure secure and sustainable food development. Furthermore are cereals and pseudocereals in general excellent substrates for growth of lactic acid bacteria (LAB) and under the right conditions, probiotic strains can be used as starter organisms when producing fermented probiotic products (Charalampopoulos et al., 2002). However, such a fermentation process requires the ability of the strain to attain high cell populations, and the adaptability and survivability of the probiotic strain in the substrate is a very important criterion.

2.4. Fermentation

Fermentation has increased in popularity over the last few years as a way to improve the nutritional aspect and taste of foods. The process has been used as a food preservation technique for centuries, and generally involves the inhibition of pathogenic bacteria by competition with beneficial microorganisms and suppression due to a low pH achieved by production of organic acids (Shiferaw Terefe & Augustin, 2020). A fermentation process can be performed either spontaneously or induced. Either way the fermentation involves a combination of a) microbial activity, b) multiple chemical reactions, and c) interactions between microorganisms and chemical compounds. The microbial activity can be described as a coexistence or selection of microorganisms by competition of nutrients. Chemical reactions lead to molecular release, synthesis, intermolecular reactions and finally, microorganisms can benefit or be inhibited by the chemical compounds.

For a fermentation to succeed, the microbial profile must display LAB as the dominant species in the niche (De Vuyst et al., 2017), as was also discussed in Paper I and III. This characteristic is widely used as a hygiene parameter of fermented products (Behera et al., 2018). LAB is also able to enhance the bioavailability of nutrients by enzymatic reactions or inhibiting the action of antinutrients such as phytates (Rollán et al., 2019). Quinoa has been used as fermentable matter to develop alcoholic and non-alcoholic plant-based beverages or baked goods (Table 1).

Organic acids that are formed because of the enzymatic activity during fermentation decrease the pH of the product (De Vuyst et al., 2017). In terms of food safety, a pH ≤ 4 is worldwide accepted as a limit. The low pH is achieved by multiple chemical reactions performed by the microorganisms synthesizing for example lactic acid (La) (both enantiomers) and acetic acid, among others (Van Kerrebroeck et al., 2016) (Paper I and III). The change in pH might also be related to the number of secondary metabolites and their capacity to increase the oxidative stress of the fermented product, which may inhibit pathogenic microorganisms (Paper III).

2.4.1. Spontaneous fermentation

Spontaneous fermentation relies on the native microbiota of the substrate and the results may therefore be unpredictable. For example, it requires long periods of time to secure the safeness of the product, which in terms of production also is a disadvantageous factor. Backslopping is an alternative method to speed up the process and shorten the fermentation time by induction of LAB dominance (Figure 3) (De Vuyst et al., 2017; Gänzle & Zheng, 2019).
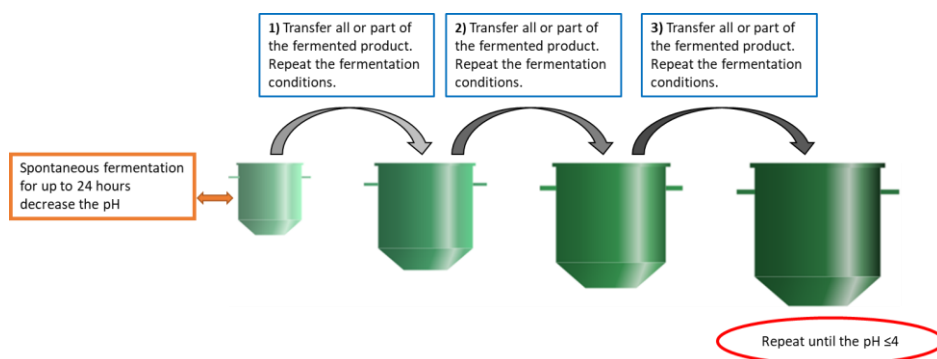
Figure 3. Visuallization of backslooping during spontaneous fermentation, figure modified from Gänzle *et al.* (Gänzle & Zheng, 2019).

There are naturally also advantageous factors associated with using spontaneous fermentations. The process is valuable and sometimes necessary to investigate and identify the autochthonous microorganisms of the substrate, and to interpret the behaviour of the microbiota, making it possible to isolate novel, viable strains (Paper II) and provide possible solutions to prevent and avoid foodborne pathogens.

The microbiota of quinoa grains has been characterized by applying spontaneous fermentation with (Franco et al., 2020; Ruiz-Rodríguez et al., 2016) or without backslopping (Vera-Pingitore et al., 2016) (Paper II). After applying backslopping daily for 10 days to ferment quinoa flour, several bacterial species were isolated and identified (underlined species were found in Paper II): *Enterococcus hermanniensis*, *Enterococcus casseliflavus*, *Enterococcus mundtti*, *Enterococcus faecium*, *Lactococcus lactis*, *Leuconostoc citrium*, *Leuconostoc mesenteroides*, *Lactiplantibacillus plantarum*, and *Levilactobacillus brevis*. Also the potential pathogens *Klebsiella oxytoca* and *Pantoea* spp., belonging to the *Enterobacteriaceae* family, were detected. (Ruiz-Rodríguez et al., 2016). In another study, applying backslopping for 8 days, the additional species were found: *Pediococcus pentosaceous*, *Latilactobacillus graminis*, *Companilactobacillus kimchi*, and *Lactiplantibacillus paraplantarum* (Franco et al., 2020).

Without the use of backslopping, *Lactiplantibacillus casei/paracasei*, *Leuconostoc lactis*, <u>*Limosilactobacillus reuteri*</u>, *Latilactobacillus sakei*, *Leuconostoc lactis*, <u>*Lactiplantibacillus plantarum*</u>, <u>*Leuconostoc mesenteroides*</u>, and <u>*Levilactobacillus brevis*</u> were isolated after 24 hrs of noninterrupted fermentation at 30 °C, immersing the quinoa seeds in MRS-broth supplemented with cycloheximide (Vera-Pingitore et al., 2016). Moreover, opportunistic pathogens of the genera *Pantoea*, *Klebsiella*, *Citrobacter*, *Enterobacter* and *Leclercia* were also found for up to 5 days of fermentation. These findings are partly in line with the results presented by Ruiz-Rodríguez *et al.* (Ruiz-Rodríguez et al., 2016), and the presence of potential pathogenic species will affect the safety of the product and possibly also the fermentation process. It can be speculated that these microorganisms originate from a contamination from water or air occurring either during the cultivation of the plant or during the production.

2.4.2. Induced fermentation

Induced fermentation is used to secure the fermentation pathways and enhances, in general, the properties and safety of the food. A starter culture, which can be a single bacterium or a consortium of several microorganisms, is added to the food matrix to give it desired properties, such as the addition of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* to milk during yoghurt production. Table 1 lists research done to ferment quinoa by using different starter cultures. The listed methodologies generally prove bacterial multiplication but lack an evaluation of the hygienic properties of the products and, in some cases, even the survivability of the starter cultures during storage time. It can also be observed that the microbiological hygiene mostly relies on the washing of the grains or on the heat treatment applied to the product prior to inoculation. However, an evaluation of the effectiveness of the cleaning and the heat treatment were in most cases not confirmed. Based on these observations, a microbiological evaluation before and after pasteurization as well as after inoculation was performed in Paper I. The findings of Paper I is supported by Kalui *et al.* (Kalui et al., 2010), who presented *Lactiplantibacillus* spp., and *Enterococcus* spp., as microorganisms that have been isolated from fermented cereals, that are associated with fermentation and that have potential to be used as starter cultures. However, the author also highlighted the health risks that may be accompanied with intake of *Enterococcus* spp. (Kalui et al., 2010). The presence of potential pathogenic bacteria in quinoa seeds after heat treatment (Paper I), led to the use of autochthonous *Lactiplantibacillus* strains as starter cultures, to evaluate their efficiency as inhibitors of the potential pathogenic bacteria from the same niche (Paper III).

Table 1. Quinoa used as matrix to develop beverages or dough fermented by the addition of starter cultures commercially aquired unless otherwise specified.

Product	Starter culture, concentration, and analysis	Fermentation conditions	Hygiene evalution	Reference
Fermentation of quinoa slurries	*Lactiplantibacillus plantarum* CRL 778 (3.0 ×10^9 CFU/mL). Control samples without starter were included. The number of *L. plantarum* CRL 778 viable cells were analyzed by plate count on MRS.	24 hrs at 30°C. The pH was ~3.9 after 8 hrs and to 3.6 after 24 hrs. pH of the control decreased to 4.7.	**NO**	(Dallagnol *et al.*, 2013)
Fermented gluten-free beer-like beverages based on quinoa	Heat treatment in several stages during the procedure. Addition of heat stable enzymes. *Saccharomycodes pastorianus* TUM 34/70, *Saccharomyces cerevisiae* TUM 177 and *Saccharomycodes ludwigii* TUM SL17 (200×10^6 cells/mL).	Fermentation temperature not reported.	**NO**	(Deželak *et al.*, 2014)
Potentially synbiotic fermented beverage with aquous extract of quinoa	*Lacticaseibacillus casei LC-1* (10^8 CFU/mL). Variable proportions of quinoa/soy were prepared and combined with soybean oil, food grade lacose, Recodam TM RS-B stabilizer, milk powder and fructooligosacharides. Thermally treated at 100 °C for 7 min, homogenized and filtrated. Mixture pasteurized at 120 °C for 20 s, and fermented during 7 or 9 hrs. MRS agar was used for the viable cell count.	37 °C until the pH decreased to 4.40-4.49 depending on the mixture.	**NO**	(Bianchi *et al.*, 2015)
Preparation of quinoa-based beverage	*L. plantarum* Q823 (1%). A mixture of 15% of quinoa flour/water was heated at 80 °C for 10 min. The beverages were stored at 4 °C after fermentation. Samples were witdrawn on day 1, 2, 5 and 7. Serial dilutions were plated on MRS agar.	30 °C for 8 hrs.	**YES** By plate count on blood agar and violet red bile glucose to detect fastidious and pathogenic bacteria, *Bacillus* spores and coliform bacteria.	(Vera-Pingitore *et al.*, 2016)
Fermented quinoa-based beverage	*L. plantarum* Q823, *L. casei* Q11 (both strains isolated from quinoa), and *Lactococcus lactis* ARH74. The quinoa was washed and dried at 60 °C for 8 hrs. The beverage contained 15% of quinoa, pasteurized at 95 °C for 10 min. The strains were individually inoculated. Viable cells were analyzed on MRS agar.	6 hrs at 30 °C. The pH decreased to around 4.	**NO**	(Ludena Urquizo *et al.*, 2017)

Improving quinoa flour through fermentation	26 autochthonous LAB such as *L. plantarum* and *Pediococcus pentosaceous* strains previously isolated from quinoa were individually used in a concentration of ca 7.0 log CFU/g, per strain. The viable cells were recorded from MRS, mMRS or SDB.	37 °C for 24 hrs. The pH decreased and varied from 3.16 to 2.60.	**NO**	(Rizzello *et al.*, 2017)
Fermented quinoa drink	*S. thermophilus, L. bulgaricus, L. acidophilus* and *Bifidobacterium* spp. Xanthan gum, sacarose-fructose and potassium sorbate were added as stabilizers to the formulation.	16 hrs at 36 °C. The pH ended between 3.60 and 3.73 depending on the formulation.	**NO**	(Maldonado Jibaja *et al.*, 2018)
Quinoa-based milk substitute	Enzymes isolated from *Bacillus subtilis*, *Aspergillus oryzae* and papain.	The enzymes were activated at 50 °C for 3 hours.	**NO**	(Jeske *et al.*, 2018)
Characterization of quinoa	*Rhizopus oligosporus* (1×10^4 spores/g). Quinoa was soaked in water for 12 hrs and steamed for 20 min at 121 °C.	Between 3 to 5 days at 30 °C.	**NO**	(Hur *et al.*, 2018)
Novel quinoa - based yogurt	*Weissella cibaria* MG1 (1×10^7 CFU/mL). The quinoa drinks were autoclaved for 15 min at 121 °C. The starch was additionally treated by heating the mixture at 50 °C for 20 min, followed for 65 °C for 90 min, and cooled at 25 °C for 40 min. The mixture was homogenized by applying high pressure homogenization. Bacterial enumeration on MRS agar plates.	30 °C for 24 hrs.	**NO**	(Zannini *et al.*, 2018)
Gluten-free cream soup based on quinoa	*L. plantarum* 1 (6 to 8% of the dough). The raw material was dry sterilized at 121 °C for 15 minutes.	35 °C for 4 days anaerobically. The pH decreased to 3.73 after 15 hrs.	**NO**	(Bolívar-Monsalve *et al.*, 2018)
Flavoring production in quinoa and wheat doughs	*L. paracasei* I1, *L. plantarum* M4 and *L. brevis* T4. Each strain was inoculated using 7-7.5 log CFU/g. LAB count was done on MRS agar.	30 °C for 48 hrs. The pH value decreased to below 4.	**NO**	(di Renzo *et al.*, 2018)
LAB and quinoa flour for manufacturing yogurt-like beverage	*Lacticaseibacillus rhamnosus* SP1, *Weissella confusa* DSM20194 as commercially available strains, and *L. plantarum* T6B10 isolated from quinoa. Initial cell density 6 log CFU/mL. The mixture was heat treated at 63 °C for 19 min.	30 °C for 20 hrs. The pH ranged between 4 to 5.	**YES** Yeast were counted on Yeast extract peptone dextrose agar supplemented with 150 ppm of chloramphenicol.	(Lorusso *et al.*, 2018)

	Internal temperature registered between 54 to 62 °C. The microbiological analysis was carried out by plate count on MRS supplemented with cycloheximide (0.1 g/L).			
Potential to use quinoa in development of a fermented spoonable vegan product.	*L. plantarum* Q823 (1% v/v). Previous to inoculation, the grains were washed and dried at 60 °C for 8 hrs. Gelatinized for 10 min and potato starch, corn starch and xanthan gum were used as stabilizers. Flavour was modified by the addition of fruits and other ingredients were also considered to improve the acceptance of the product.	30 °C for aproximately 6 to 8 h. The pH decreased to below 4.	**YES** Total aerobic, mesophilic microbes, yeast and molds, and coliforms were counted.	(Väkeväinen *et al.*, 2020)
Fermented quinoa-based beverage **(Paper I)**	*L. plantarum* DSM9843 (10^{10} CFU/mL). The beverage was heat treated at 65 °C for 30 min. Viable cells were enumerated from Rogosa agar.	30 °C for 48 hrs, at anaerobic conditions. The pH decreased to below 4.	**YES** Total aerobic count and *Entobacteriaceae* were counted, isolated and identified. Yeast and mould were counted.	(Canaviri Paz *et al.*, 2020)
Quinoa used for development a fermented beverage	*Bifidobacterium* sp., *Lactobacillus acidophilus*, and *S. thermophilus* (in total 10^8 CFU/mL). The quinoa grains were washed and dried at 60 °C for 8 hrs followed by milling. The quinoa beverage was heat treated at 95 °C for 10 min and cooled at 20 °C. LAB was recorded by plate count on M17 and MRS.	37 °C for 6 hrs. The registered pH was 4.19.	**NO**	(Karovičová *et al.*, 2020)
Quinoa sourdough for gluten free muffins	*L. plantarum* ATCC 8014 (3.2 log CFU/g). The quinoa flour was mixed with water in a 1:1 ratio. A spontaneous fermentation was used as control.	37 °C for 24 hrs. The pH values were not reported.	**NO**	(Chiş *et al.*, 2020)
Yogurt fortified with quinoa flour	3 combinations of microorganisms were used: *S. thermophilous* and *L. brevis* NRRLB 4527 (1:1*), S. thermophilus* and *L. reuteri* NRRLB-14171 (1:1), *S. thermophilus* and *Latilactobacillus curvatus* NBIMCC 3452 (1:1). Buffalo milk was heated at 90 °C for 10 minutes and cooled to 42 °C. The viable cell counts were done on M17 and MRS agar.	42 °C Fermentation time reported as a function of the pH. The pH registered was 4.6-4.7	**YES** Coliform counts were received from VRBD agar according to the APHA method (2001)	(Mabrouk & Effat, 2020)

Probiotic red quinoa drink for celiacs and lactose intolerant people	*L. plantarum* CECT 220 and *Bifidobacterium longum* CECT 4551. Initial concentration of 6 log CFU/mL. The red quinoa grains were milled and mixed with water. The mixture was pasteurized at 80 °C for 20 min. The viability of the starter cultures were determined on MRS agar.	37 °C for 24 hrs. The pH decreased from 4.30 to 3.40.	**YES** Mould and yeast determination using Petrifilm™ yeast and mould count plates. The plates were incubated for 48 to 72 hrs for yeast and for 72 to 140 hrs for moulds in aerobic contiditons at 25 °C.	(Cerdá-Bernad *et al.*, 2021)

A pH value ≤ 4 was used as a measure of satisfactory fermentation in most studies previously listed (Table 1). However, it is not obvious that the starter culture alone is responsible for the decrease in pH and formation of La since, for example, *Enterococcus* strains are also considered producers and acid resistant (Haghshenas et al., 2014). To include a microbiological analysis along with pH measurements might therefore be preferrable as a hygiene measure of fermented food products.

2.5. Key points

✓ Efficient heat treatment has not been properly developed for plant-based products.

✓ Commercial starter cultures will not always be a suitable inoculum due to the characteristics of the niche.

✓ A decrease in pH or increase in concentration of La after fermentation must be revised in terms of safety since potential pathogenic microorganisms from the genera *Enterococcus* is acid resistant and can produce La.

✓ Quinoa seeds can be used as functional foods. It is proven that quinoa seeds can be a source of potential probiotics and be used as matrix to develop fermented products.

✓ The bacterial content must be evaluated for plant-based beverages to ensure the hygiene and safety of the products.

3. Bacterial diversity of quinoa seeds

To date information describing the native microbiota of cereals, pseudocereals, and grains is scarce. In this thesis, microbiota characterization of raw quinoa seeds was conducted in Paper II, with special attention to potential pathogens as well as to beneficial strains with specific properties. To set and standardize the study designs, experimental trial 1 and 2 (described below) were performed prior to the implementations. The potential pathogenic bacteria detected during development of the quinoa-based beverage in Paper I was of high relevance, indicating the need of a meticulous microbiological analysis. The results of Paper II presented valuable information of microbial properties that might be used to optimize the fermentation process.

3.1. Autochthonous microbiota of quinoa seeds

Bolivian white quinoa grains from the two trademarks Kung Markatta (Paper I and II) and Saltå Kvarn (Paper III and IV) were purchased from retails in Lund, Sweden. The experimental procedures were designed to stimulate growth of bacterial species that normally are in minority on the grains and to minimize the disturbance of the microbial population during fermentation i.e., spontaneous fermentation without backslopping were applied under static and initial anaerobic conditions.

3.1.1 Microbial cultivation

Live microorganisms and the number of cells were isolated and recorded respectively from the quinoa samples. With the aim of obtaining an as complete picture as possible of the viable microbiota present on the quinoa seeds, several different cultivation media were used. Violet red bile dextrose agar (VRBD) was chosen as it is selective for *Enterobacteria*. Tryptic soy agar (TSA) was used for total aerobic count, and it was also used for subculturing of, for example, *Enterobacteriaceae* and *Staphylococcus* spp. Furthermore, Malt extract agar (MEA) was chosen as a medium for enumeration of yeast and moulds and Rogosa agar as being selective for lactobacilli growth, when supplemented with acetic acid to decrease the pH and thereby inhibit growth of other LAB (Paper I-IV).

Experimental trial 1

Induced and spontaneous fermentation were performed in parallel to study compositional changes of the microbiota. The quinoa grains were mixed with cold sterile water in a proportion of 1:3 (w/v) and poured into glass bottles filled to the maximum capacity (500 mL flasks) to reduce the amount of oxygen. One sample was prepared for each type of fermentation. For the induced fermentation, *L. plantarum* 299v (=DSM9843) was added (10^{10} CFU) as starter culture. The mixtures were fermented at 30 °C for 24 hours. The pH was controlled at each sampling point (Table 2). Samples of quinoa seeds were withdrawn before fermentation and after 6, 12, and 24 hours. 3 grams of quinoa seeds were mixed with 27 mL of sterile peptone water as described in Paper I-IV. The samples were homogenized using a Stomacher at high speed for 1.5 minutes. Serial dilutions were performed until 1/10000 DF, and from each dilution 100 µL were plated in duplicates on VRBD, TSA, MEA, and Rogosa agar. Viable cells were visually examined but not isolated (Figure 4).
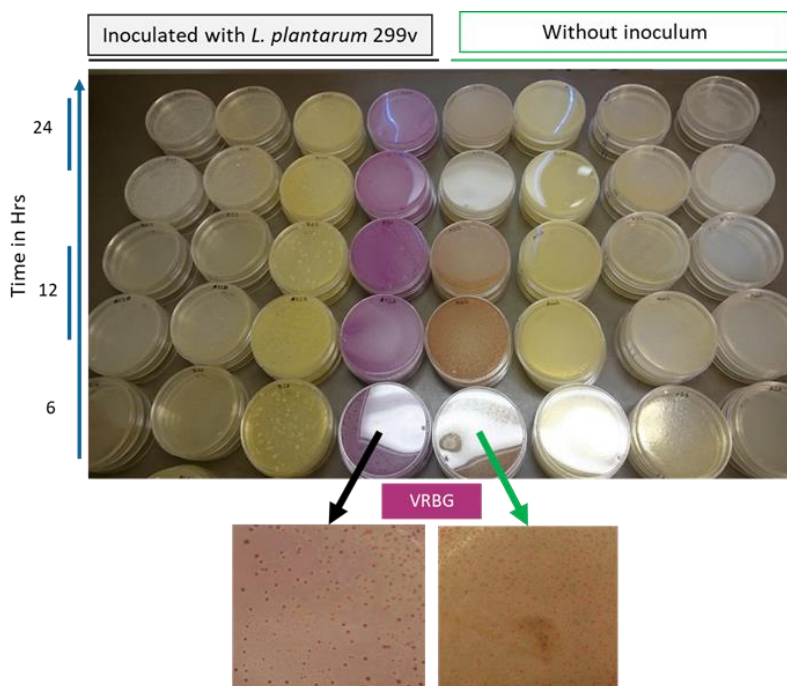
Figure 4. An overview of viable cells using plate counts. No difference was observed between induced and spontanelusly fermented quinoa seeds.

The number of colonies were uncountable on all agar plates. The most remarkable result was the high number of *Enterobacteriaceae* even after 24 hours of fermentation in the presence of *L. plantarum* DSM9843. The results were not

expected since most of the reviewed studies (Table 1) did not report the presence of potential pathogenic bacteria at such high concentrations after 24 hours of induced fermentation.

Table 2. pH values registered at 0, 6, 12 and 24 hours of induced (*L. plantarum* DSM9843) and spontaneous fermentation of quinoa seeds.

Time (Hrs)	Fermentation	
	Induced (*L. plantarum* DSM9843)	Spontaneous
0	6.43	6.42
6	4.20	6.40
12	4.27	5.22
24	4.01	4.58

There are several sources from which the contaminating microorganisms can origin, e.g. from water, air, or from the grains (Los et al., 2018). The drinking water in Sweden is of very high quality and should not cause contamination during washing of the seeds, but to confirm that the pipes in the lab were not the cause, aliquots of the tap water were collected and plated on VRBD. No viable cells were observed (data not shown). That the microorganisms should originate from the air in the laboratory environment was not considered likely. The most probable source of potential pathogens is therefore the seeds themselves, which makes it very important to control the hygiene of the product at different points during the production chain and exportation.

Experimental trial 2

A mixture of quinoa seeds and water (1:2 w/v) where used to accomplish induced fermentation (n=2) and spontaneous fermentation (n=2) at 30 °C for 8 days (Figure 5). The experimental design was set to evaluate the effect of air inflow on the fermentation process while opening the bottles for sampling. One bottle was therefore opened at regular intervals for sampling (OIf-1 and OSf-1), meanwhile one bottle was kept closed until the end of the fermentation process (CIf-2 and CSf-2). Samples were withdrawn from OIf-1 and OSf-1 at 0, 12, 24 hours and continuously once per day until the eighth day. Plate count was performed as previously described, excluding MEA. Four colonies were randomly picked from each agar plate and purified twice. The cells were stored in freezing media at -80 °C (Siv Ahrné et al., 1989) for further analysis.
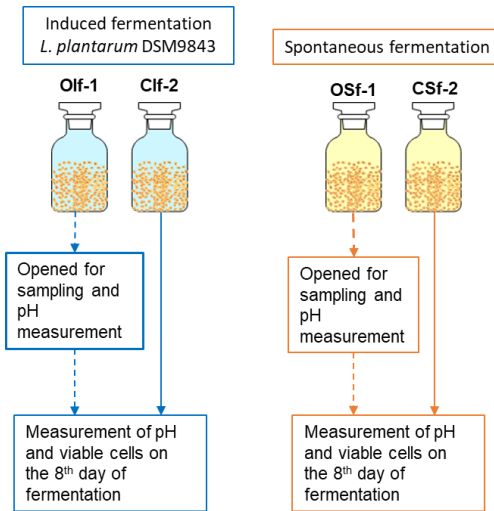
Figure 5. Experimental design to evaluate the effect of air inflow on the fermentation process while opening the bottles.

After 48 hours of induced fermentation, the pH decreased from 6.43 to 3.89 (OIf-2), and remined constant until 192 hours (8 days). At this point, the closed bottle (CIf-2) was opened, and the pH value was 3.93. For the spontaneous fermentation, the initial pH was 6.42 and it decreased to 4.10 after 144 hours (6 days) (OSf-1). After 8 days the closed bottle (CSf-2) was opened, and the pH was 4.12. The number of viable cells and changes in pH values were recorded (Table 3).

Table 3. pH and number of viable cells (Log CFU/g).

Time (hrs)	Spontaneous (Log CFU/g)				Induced (Log CFU/g)			
Opened bottles	pH	Rogosa	TSA	VRBD	pH	ROGOSA	TSA	VRBD
0	6.42	≤1	≤1	≤1	6.43	7.23	≤1	≤1
12	6.40	≤1	1.5	2.50	4.27	9.13	10.0	≤1
24	5.22	7.70	8.47	6.20	4.01	9.43	10.4	≤1
48	4.58	9.32	9.27	6.80	3.89	8.23	10.1	≤1
72	4.31	9.12	9.09	≤1	3.91	9.35	10.4	≤1
96	4.27	8.95	8.92	≤1	3.92	8.85	9.89	≤1
120	4.19	9.00	8.97	≤1	3.89	8.54	9.52	≤1
144	4.16	8.91	8.84	≤1	3.87	8.40	9.34	≤1
192	4.10	9.00	9.13	≤1	3.86	8.88	10.3	≤1
Closed bottles								
192	4.12	8.91	8.92	≤1	3.93	7.95	7.92	≤1

A total of 140 isolates from different times points, divided as 68 from OIf-1, and 72 from OSf-1 were collected. Four colonies were isolated from CIf-2 and from CSf-2 respectively. The isolates were analysed by 16S rRNA gene sequencing and Sanger sequencing.

Based on the experimental trial 2, it was concluded that the air inflow in connection with the opening of the bottles did not have any remarkable impact on pH or viable cell counts, except for growth on TSA during induced fermentation. In Paper II, the results showed that fermentation will stimulate the growth of the autochthonous microorganisms including both potential pathogens and LAB, probably jeopardising the backslopping technique, since backslopping relies on the native microbiota. The present research indicates a risk of using backslopping within 0 and 24 hours of fermentation of quinoa seeds, due to the high concentration of *Enterobacteriaceae* spp. When all or parts of the fermented matter is mixed with new, unfermented substrate, the concentration of viable LAB cells is not a warranty of safeness as coexisting, potential pathogens might be simultaneously transferred during the backslopping process (Figure 6).
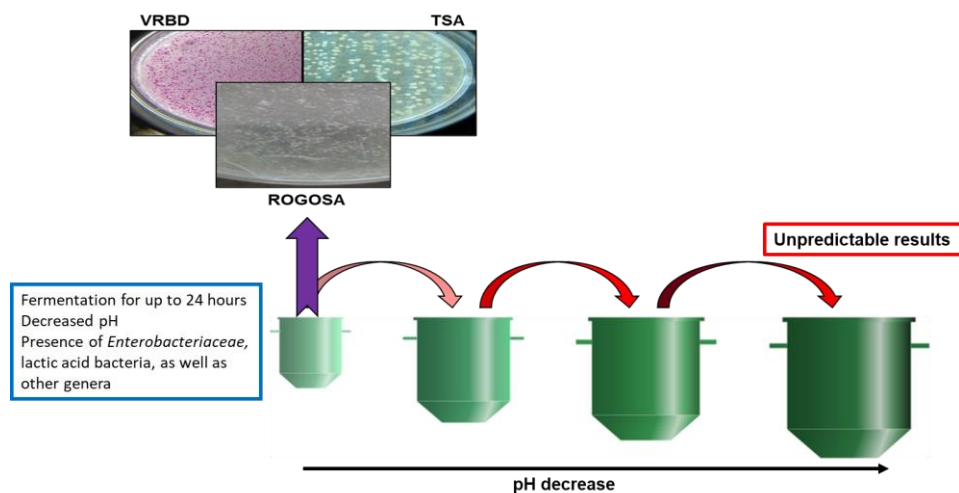
Figure 6. A schematic figure of the risks associated with the backslopping process.

As is commonly known, the results of microbial cultivation are dependent on the dilution applied and in Table 4, the serial dilutions used per agar through time for Paper I, II, III and IV are summarized. However, it is also known that plate cultivation has limitations associated with existence of unculturable bacteria and dormant cells, which will restrain the possibility to analyse the complete microbial community. It is however conceivable to isolate the dominant, culturable organisms. Using plate cultivation as a first approach is therefore important as a tool for isolation of new microorganisms.

Table 4. Dilutions of samples for viable counts to isolate dominant, viable cells of the microbiota on the quinoa grains and in fermented quinoa-based beverage.

Days	Dilution factor used for VRBD 🔴, TSA* 🟤, and Rogosa 🟠																				
	0	0	1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10
0	🟤	🟠	🟤	🟠	🔴	🟤	🔴		🔴		🔴										
1					🔴		🔴		🔴		🔴	🟤	🟤	🟠	🟤	🟠					
2					🔴		🔴	🟤	🟤		🟤				🟤	🟠	🟤	🟠		🟠	
3					🔴	🟤	🔴	🟤	🔴						🟤	🟠	🟤	🟠	🟤	🟠	
4			🔴		🔴	🟤	🔴									🟠		🟠		🟠	🟠
5			🔴	🟤	🔴	🟤						🟤		🟤			🟤	🟠		🟠	🟠
6	🔴		🔴	🟤	🔴										🟤		🟤	🟠		🟠	🟠
7	🔴		🔴	🟤											🟤		🟤	🟠		🟠	🟠
8	🔴		🔴	🟤											🟤		🟤	🟠		🟠	🟠

*The dilutions used for TSA were also applied for MEA.

3.1.2. Conventional genetic identification

For a proper genetical identification, a combination of methods may be needed to approach the identification from both a general point of view and, as in this thesis, by using more specific molecular methods to discriminate between closely related species, such as native *Lactiplantibacillus* species.

Some of the more molecular methods require pure cells without any contamination. After multiplication of the cells, the cells were purified as explained in Paper I, II and IV. The DNA was extracted from the cells by breaking the cell wall during shaking with glass beads (M. -L Johansson et al., 1995). The same process was used to prepare the stool samples before DNA extraction (Paper IV).

The extracted DNA from the viable cells isolated from Experimental trial 2, and Paper I and II were identified molecularly. There are several DNA based methods that can be used for indication of microorganisms based on the polymerase chain reaction (PCR), which copies DNA fragments. Briefly, PCR uses primers, which are short oligonucleotides complimentary to the DNA sequence in the target gene, a DNA polymerase enzyme, a buffer, deoxyribonucleotides (dNTPs) and varying temperatures to amplify the target gene.

In bacterial taxonomy, the 16S rRNA gene is widely used as a measure of evolutionary relationships (Thorne et al., 1998). The gene consists of both conserved and variable regions that can be used for identification purposes. In this thesis, the primers ENV1 (5´-AGA GTT TGA TII TGG TCT AG-´3), where I represents Inosine, and ENV2 (5´-CGG ITA CCT TGT TAC GAC TT-´3) (Brosius

et al., 1978) have been widely used (Paper I and II) to copy approximately the whole 16S rRNA gene. However, the primers present limitations when used for discrimination between closely related species such as *L. plantarum, L. pentosus* and *L. paraplantarum*, which share 99.9% 16S rRNA gene sequence similarity.

After the 16S rRNA gene has been amplified, Sanger sequencing can be used to determine the composition of the bases in the gene (Paper I and II). Sanger sequencing, or the chain termination method, was developed in 1977 and is based on the principle of exchanging dNTPs with dideoxynucleotides (ddNTPs), which lack the hydroxyl group necessary for incorporation of the next nucleotide in the DNA strand (Sanger et al., 1977). This will result in several fragments differing with one nucleotide pair in length. By using different tags on the different nucleotides, they are then translated and compared to known DNA sequences in a database, and an identity of the microorganism can be determined. Table 5 presents the microorganisms identified from Experimental trial 2, and for Paper I and II, the identities are presented in Table 3 and Table 2 in the respective publication.

Table 5. Identified bacteria from experimental trial 2.

Induced fermentation with *L. plantarum* DSM9843		Spontaneous fermentation	
Olf-1	**Clf-2**	**OSf-1**	**CSf-2**
Enterobacter spp.	*E. hirae*	*E. durins*	*E. hirae*
Staphylococcus spp.	*E. mundtti*	*E. mundtti*	*L. lactis*
L. plantarum	*L. plantarum*	*E. hirae*	*P. pentosaceous*
P. pentosaceous		*L. plantarum*	*L. plantarum*
		L. pentosus	*L. pentosus*
		P. pentosaceous	*P. pentosaceous*

However, there are also limitations of Sanger sequencing such as a relatively high cost, it is work intense and generally generate low quality in the first and last bases. In this thesis, those bases were corrected using BioEdit, a confinable computer program (Hall, 1999).

In a further attempt to discriminate between *L. plantarum and L. pentosus*, specific molecular regions in their genome were targeted. The method was originally developed by Torriani *et. al*. (Torriani et al., 2001), and was targeting the recA gene using a multiplex-PCR assay. Another method to differentiate between the species was developed by Berthier *et al*. (Berthier & Ehrlich, 1998), and targeted the 16S rRNA region with specifically designed primers. Both methods have previously been found efficient. However, in this thesis it was observed that the method developed by Berthier *et al*., was more effective in discriminating between the autochthonous *Lactobacillus* spp., isolated from quinoa than the method by Torriani *et al*.

To give supportive information in an effort to discriminate between the lactobacilli species, randomly amplified polymorphic DNA (RAPD) was performed in Paper II.

RAPD uses short oligonucleotides that randomly amplifies DNA fragments in an identical pattern for identical species, while the patterns differ between distantly related species (Figure 7) (M. -L Johansson et al., 1995). The resulting band patterns can be visualized by gel electrophoresis. Unfortunately, RAPD has disadvantages in being a sensitive method, and the results depends on several factors (Taleb-Hossenkhan, 2020). On the contrary, RAPD is fast to perform, and the results can be analysed and interpreted almost immediately if type strains are used as references. In Figure 8, besides the autochthonous *Lactiplantibacillus* spp., type strains were used as references (Paper II).
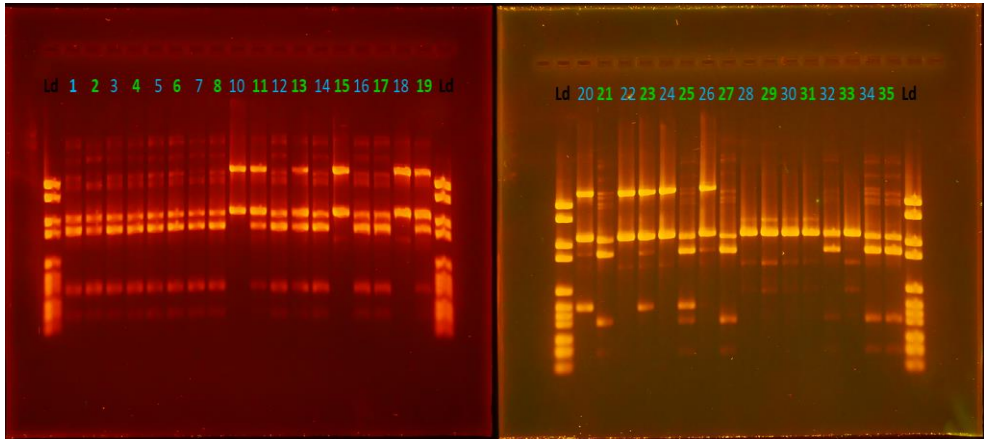
Figure 7. Representative bands patterns of *Lactiplantibacillus* strains isolated from quinoa seeds in 2016.
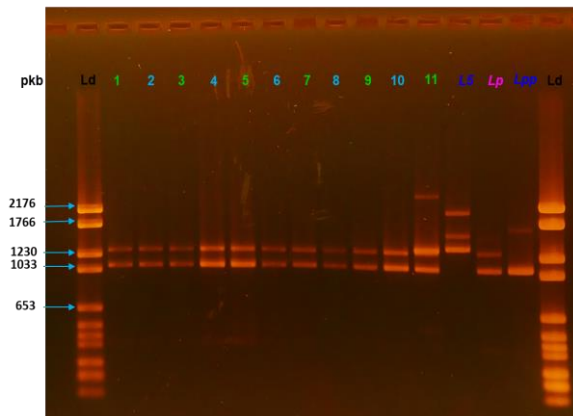
Figure 8. Representative band patterns of the *Lactiplantibacillus* strains isolated from quinoa seeds in 2017 compared with type strains, where *L5* stands for *L. pentosus*, *Lp* for *L. plantarum* and *Lpp* for *L. paraplantarum*.

3.1.3. Enzymatic characterization

Information about which substrates a bacterium can use as nutrients is significant, especially in the quest to find new LAB with possibilities to be used as starter cultures. API 50CH is a commercial kit which contains 49 different carbohydrates and is designed to phenotypically characterize lactobacilli strains (Paper II). Since the technique is based on enzymatic actions leading to chemical reactions, the microorganisms tested must be viable. Single isolates are tested in each kit and therefore, the results correspond to that specific strain. The results of the assay can be obtained during the first 24 hours; however, the analysis should be followed for 2 days, or as recommended by Nigatu *et. al*. (Nigatu et al., 2000), for up to 7 days. To decide which isolates to be tested in this thesis, the band patterns from RAPD were evaluated (Figure 7, Figure 8), and the cells with the same band pattern were chosen. The higher the number of carbohydrates that the *Lactiplantibacillus* spp., can ferment, the greater capacity it may have in the fermentation process when being used as a starter organism.

In Paper II, methyl gallate degradation and gallic acid decarboxylation were used to evaluate the tannase enzymatic capacity of the autochthonous *Lactiplantibacillus* strains isolated from quinoa by a colorimetric assay (Osawa et al., 2000; Osawa & Walsh, 1995). The assay was of importance since species such as *Bacillus* spp., (Aguilar-Zárate et al., 2015; Jana et al., 2013; Raghuwanshi et al., 2011), *Enterococcus* spp., (Goel et al., 2011), and species from the genera *Enterobacter* (Sharma & John, 2011) and *Klebsiella* express tannin degradation capacity and they were identified as part of the quinoa microbiota and are also to some extent associated with hazardous mechanisms for human health (Paper II) (Ruiz-Rodríguez et al., 2016) Oscarsson et al., 2020; Townsend et al., 2007). Because of the tannin degradation capacity, the microorganisms can use the plant as a nutrient resource while benefiting the plant by offering protection against environmental factors (Sharma, 2019).

The results reported in Paper II also show that tannase activity can be expressed by *Lactiplantibacillus* spp., isolated from quinoa, but not by all strains. For the beneficial of humans, the metabolization of tannin molecules to smaller molecules decrease the tannin activity as antinutrients. Tannins in higher concentrations are responsible for precipitation of proteins, decreasing the absorbance in the body. However, the increase in concentration of gallic acid or pyrogallol as products of tannin degradation are advantageous for human health (Fei et al., 2017).

3.2. Novel *Lactiplantibacillus* spp.

From the lactic acid bacteria isolated from spontaneously fermented quinoa dough plated on Rogosa agar, 51.8% were *Lactiplantibacillus* spp., while 48.1% were identified as *Pediococccus* spp. From the bacteria being identified as *Lactiplantibacillus* spp., four strains were selected for further evaluations. Two of those strains were identified as *L. plantarum* and two were suspected to be *L. pentosus*. Bacteria belonging to the former genus *Lactobacillus* are considered "generally regarded as safe" (GRAS) and has to a great extent and for a long time been used in different probiotic products or as starter cultures in the food industry.

3.2.1. *Lactiplantibacillus plantarum*

Strains from the *L. plantarum* species are either obligate homofermentative, i.e., able to ferment hexoses into La, or heterofermentative and able to ferment hexoses into La, acetic acid and/or ethanol and carbon dioxide. Strains belonging to the *L. plantarum* species are stress tolerant and can survive the passage through the human gastrointestinal tract (M. L. Johansson et al., 1993). Furthermore, they can ferment several carbohydrates and are found as commensals in fermented foods or as part of the intestinal microbiota (S. Ahrné et al., 1998; Molin et al., 1993).

The two isolated *L. plantarum* strains from fermented quinoa dough will be further referred to as *L. plantarum* 5 and *L. plantarum* 9. *L. plantarum* 5 and *L. plantarum* 9 are described as obligate homofermentative and can adapt to anaerobic and aerobic conditions. They were found to be able to grow on Rogosa, TSA and MEA. The ability of the colonies to grow on MEA might be explained by the fact that the composition of nutrients is similar to what the bacteria experienced on the quinoa seeds. Furthermore, the strains expressed DL-lactate racemase activity (Paper III) which can catalyse the conversion between enantiomers of La.

3.2.2. *Lactiplantibacillus pentosus*

L. pentosus is known for its ability to withstand environmental stress. It has even been found to get enhanced probiotic properties if pre-treated with acid (Cubas-Cano et al., 2019). Additionally, it has been found to reduce contamination of the mycotoxins Zearalenone in food (Sangsila et al., 2016). A special characteristic for strains belonging to *L. pentosus* is their capacity to ferment D-xylose. This characteristic is used specially to distinguish *L. pentosus* from other LAB. However, this thesis has identified *L. plantarum* strains also expressing the capacity to degrade D-xylose (Paper II). Overall, the information that can be found about *L. pentosus* is limited compared to the information available for *L. plantarum*, mainly because it is difficult and complex to discriminate between the species.

The two isolated *L. plantarum* strains from fermented quinoa dough that was suspected to belong to the species *L. pentosus* are further referred to as *L. plantarum* 3 and *L. plantarum* 10. The two strains *L. plantarum* 3 and *L. plantarum* 10 possess the D-xylose degrading capacity and were chosen for further investigation. Both strains have the capacity to lower the pH and resist acidic environments. The strains also have the capacity to convert D (-)-La to L (+)-La due to its DL-lactate racemase activity (Paper II and III). The presence of the xylose degrading enzyme can be related to the content of xylose in quinoa stalks, which is for example higher than in corn or wheat straw (Salas-Veizaga et al., 2017).

3.3. Microbial composition of quinoa beverage

Based on the findings presented in Paper I and II it is suggested that the potential pathogenic bacteria found in the quinoa-based beverage (Paper I) came from the quinoa seeds. The study by Rodriguez *et al*., (Ruiz-Rodríguez et al., 2016), listed in Table 1, reports the presence of *Klebsiella oxytoca* and *Pantoea ananatis* for two consecutive days during spontaneous fermentation of quinoa sourdough using backslopping. The findings are in line with the results of Paper II in the present thesis. To the best of our knowledge this is the first study focusing not only on the benefits of quinoa seeds, but also on the hygiene risks with developed products based on quinoa. Despite the food hygiene risks just mentioned, neither the physicochemical nor the nutritional properties of quinoa seeds should be neglected. On the contrary, it is for example important to mention that the quinoa-based drink developed in Paper I exhibit great stability without stabilizers or additives and *L. plantarum* DSM9843 survived for up to 28 days with no additional resources except what was offered by the quinoa. The fermentation process was also found to inhibit *Enterobacteriaceae* to a great extent, but *L. plantarum* DSM9843 did not completely dominate the bacterial composition after fermentation as viable *P. pentosaceous* and *E. mundtti* were detected (Paper I).

The induced fermentation process is naturally dependent on the starter culture, and different bacteria will be more suited for specific environments. Using bacteria isolated from the raw materials as starter cultures in fermentation processes have been previously performed (Carrizo et al., 2016; Franco et al., 2020; Rizzello et al., 2016), based on the hypothesis that the microorganisms are already adapted to the plant environment and can metabolize the nutrients from the raw material. In an effort to improve the hygiene quality and flavour of the quinoa-based drink (Paper I), autochthonous microorganism isolated from quinoa (Paper II) were used as starter cultures in Paper III. The microbiota composition of the quinoa-based beverages were mapped using NGS with Illumina MiSeq and the flavour of the beverages was improved by toasting the quinoa seeds (di Renzo et al., 2018), prior to preparation of the beverages (Paper III).

3.3.1. Genetic characterization

The development of NGS revolutionized the field of sequencing. Previously, sequencing methods had been expensive, time consuming and inefficient but with introduction of the NGS technology, sequencing became much more available and today, Illuminas technology is probably one of the most used technologies in the world. Illumina sequencing, which has been used in Paper III and IV in this thesis, is based on sequencing by synthesis (SBS). The sequencing procedure includes four main steps in which DNA is prepared in the first stage. The DNA fragment of interest is amplified, and adapters are added to the sequence to facilitate the next step. The DNA sequences are loaded on a flow cell where the reaction occurs. The flow cell is coated with short oligos that are complementary to the adaptors on the DNA fragments, and when the DNA has bound, they are amplified into clusters through bridge amplification. Then, the sequencing occurs where bases are detected as a fluorescent signal when they are incorporated to the growing DNA strand. The obtained sequences are then analysed and compared to reference genomes. Several packages are available for analysing with a proper improvement of sequencing data, for example Qiime and Mothur (López-García et al., 2018).

The composition of the fermented quinoa-based beverage was analysed using NGS with Illumina MiSeq. The results showed that the lactobacilli dominated the samples to variable extents. For example, *L. plantarum* 3 and *L. plantarum* 10 dominated the niche more efficiently (approximately over 90%) than *L. plantarum* 5 and *L. plantarum* 9 (approximately less than 55%) (Paper III), indicating that not all lactobacilli behave the same, and even when an exponential growth of the strains was registered, not all dominated the niche. A correlation between the results from the cell culturing and NGS (Figure 9) was discovered, where cell culturing was shown to be a reliable method, and it is useful when applying correct sample concentrations and for researchers from developing countries.
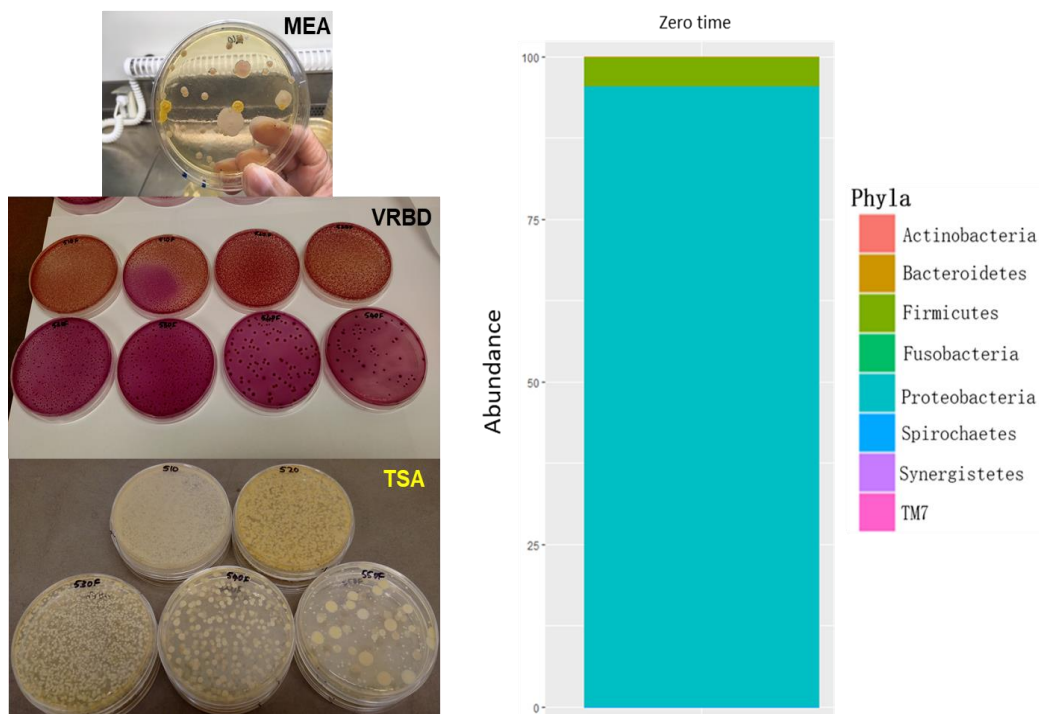
Figure 9. The figure is illustrating a correlation between plate count (left) and NGS (right), where a high concentration of potential pathogens are found before starting the fermentation.

3.4. Key points

✓ The characterization of the microbiota of quinoa grains is a new area of research. The isolated bacteria from quinoa seeds were identified at genotypical and phenotypical level.

✓ Based on the findings, it is demonstrated that the autochthonous microbiota of quinoa seeds contains high numbers of viable potential pathogenic bacteria.

✓ Until now, potential pathogenic bacteria in quinoa seeds have not been discussed in relation to food safety, mainly because it is assumed that possible contamination originates from air or water and is neutralised by heat treatment. Unfortunately, heat resistant *Enterobacteriaceae* were still present in substantial concentrations after pasteurization. The findings in Paper I and Paper II were therefore of significant importance.

- ✓ The number of viable *L. plantarum* DSM9843 cells as well as pH and levels of D-La and L-La stayed constant over time.

- ✓ The fermented quinoa beverage was homogenic and no sedimentation was observed. Furthermore, the *L. plantarum* DSM9843 cells survived during storage time and no addition of carbohydrates was needed.

4. Synergy between polyphenols and native *L. plantarum* spp.

Plant-based beverages have become an interesting source of nutrients in which the increase in bioavailability of micronutrients, such secondary metabolites, can be obtained by induced fermentation. Autochthonous *Lactiplantibacillus* strains isolated from quinoa seeds (Paper II) were used as starter cultures to ferment a quinoa-based beverage. The synergy between the inoculums and the polyphenolic compounds during fermentation is not completely understood, but an approximal interpretation and explanation is suggested in Paper III.

4.1. Chemical characterization

The identification of secondary metabolites in this thesis has focused on polyphenolic compounds from quinoa, which are described in Chapter 2 (Section 2.2.2). These phytochemicals are part of the quinoa seeds and are found in free form or bound to the organic matrix. Some attempts have been made to increase the amount of free and available polyphenols in quinoa. Extraction methods using extreme basic or acidic conditions with sodium hydroxide or chlorohydric acid at concentrations of 10M or 2M (Tang et. al., 2016) or enzymatic reactions with, for example, alfa-amylase (Chen et.al 2015) to release the bound secondary metabolites have been used.

4.1.1. High-performance liquid chromatography

Nowadays, high-performance liquid chromatography (HPLC) is the analytical technique that is most used worldwide for the identification and quantification of molecules. The separation of compounds is based on a stationary phase and on a mobile phase, as any other chromatographic technique (Lozano-Sánchez et al., 2018).

Secondary metabolites from food matrices have been identified based on their molecular properties, such as solubility, and polarity, just to mention some. Reverse phase liquid chromatography (RPLC) is based on the principle where hydrophobic

compounds are well retained. Secondary metabolites with higher polarity will be detected first followed by the less polar ones. The metabolites must therefore be soluble in polar organic solvents, such as acetonitrile, methanol, or water which are also used as mobile phase.

4.1.2. Sampling pre-treatment

The quinoa samples were collected before fermentation (zero time), after fermentation (2 d), and during storage time (14, and 28 d), respectively, and stored at -20 °C in darkness until analysis. The samples were thawed at 4 °C in darkness overnight and centrifuged to separate the liquid part from the solids (Paper III). The samples were protected from the light to avoid possible degradation. The samples were filtered using a 0.2 µm pore diameter, as is described in Paper III. The filtration process prevents possible damage to the column, used as stationary phase. The samples were collected and analysed in triplicates.

4.1.3. Polyphenolic compounds identification

The identification and quantification of the polyphenolic compounds were performed using an Agilent Technologies HPLC 1100 series assembled as is described in Paper III with UV/VIS detection. The selected wavelengths were 280, 325 and 360 nm.

As stationary phase, the column Xselect C-18 (Phenomenex, USA) was used due to its pH and temperature resistance (pH range 1-11, and maximum temperature of 60 °C). The HPLC thermostat was settled at 30, 35, 40 and 50 °C on previous experimental trials. Adequate retention time reproducibility and peak shape were found at 30 °C using acidified methanol and water as eluent. Furthermore, the column can be adapted to a mass spectrometry system, therefore the retention time per metabolite can be replicated, facilitating the identification of the polyphenols by molecular weight. The identification of the phenolic and flavonoid compounds will be addressed in future studies.

As an overview of the total concentration of polyphenols (max. 280 nm), and flavonoids (max 360 nm), approximal calculation of the total concentrations were expressed as mg/100g equivalent of gallic acid for total polyphenols, and as equivalents of quercetin for flavonoids (Figure 10). The results showed no statistically significant variation in the content of total phenolic compounds from quinoa-based beverages fermented with *L. plantarum* 3, *L. plantarum* 5, *L. plantarum* 9 and control, but differences were found after fermentation using L. *plantarum* 10. In the case of total flavonoids, none of the calculated results presented a significant variance (see Table 4 in Paper III).
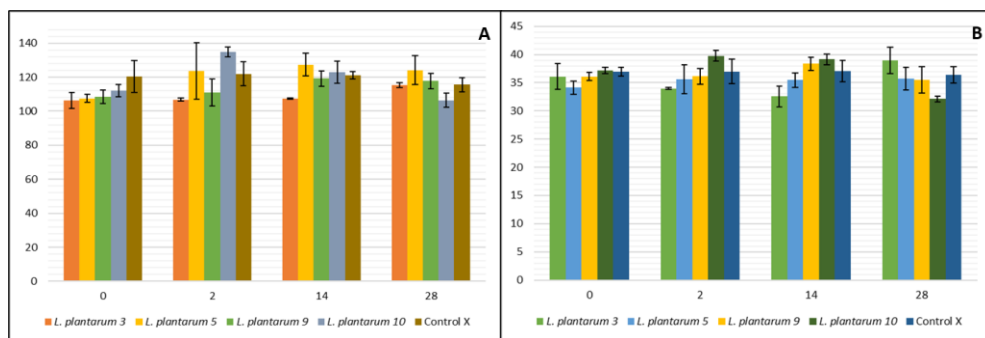
Figure 10. Summary of the content of total phenolic compounds (A), and total flavonoids (B).

4.2. Effect of the fermentation on polyphenols and flavonoids

Gallic acid, catechin and quercetin 3O-glucoside, just to mention some of the identified compounds, were found in different concentrations (Paper III). Altered concentrations of polyphenols caused by the native *Lactiplantibacillus* strains indicate that the polyphenolic compounds are metabolized during fermentation (Paper III). Firmicutes was the phylum dominating the products for two of the four tested autochthonous *Lactiplantibacillus* strains after fermentation. The other two strains had a higher presence of Proteobacteria after fermentation and based on the significant decrease of 4-HBA it can be argued that the native microbiota may be involved in the metabolism of the polyphenolic compounds during the fermentation process. Additionally, a significant decrease of gallic acid was found in the control group, which was spontaneously fermented, further strengthening the assumption that the native microbiota is involved in the degradation of phenolic and flavonoid compounds. However, some polyphenols change in concentration only in the samples fermented with specific lactobacilli strains, indicating that different inoculums had different effects on polyphenols. Therefore, to acknowledge that the changes in the content of phenolic and flavonoid compounds during fermentation is exclusively due of the enzymatic action of the bacterial strains used as starter culture might not be completely true. The results found in the study rather indicate that both the native microbiota and the starter culture strains participate in the process.

4.3. Key points

- ✓ Both the native microbiota and the microorganisms used as starter cultures induce changes of secondary metabolites during fermentation.

- ✓ The microbiota of the unfermented quinoa-based beverage constituted of more than 90% of the Protobacteria phylum.

- ✓ All tested strains multiplied during fermentation, but *L. plantarum* 5 and *L. plantarum* 9 were not able to dominate the niche.

- ✓ High-performance liquid chromatography was used as an analytical tool for identification and quantification of phenolic and flavonoid compounds in unfermented and fermented quinoa-based beverages.

5. Effects on microbiota composition by a native *L. plantarum* strain

To categorize a LAB as probiotic, high standard requirements need to be achieved. It is for example important that the probiotic strain is resistant to the harsh conditions in the gastrointestinal tract, are safe and confer a health benefit to the consumer (FAO/WHO, 2002). The effect the potential probiotic has on the gut microbiota is also an interesting feature to study. Furthermore, it is an advantage if the potential probiotic strain is easy to cultivate and an efficient fermenter. Based on the strain characteristics investigated and previously described (Paper II and III), the probiotic potential of the novel autochthonous *L. plantarum* 3 was tested and evaluated in healthy volunteers (Paper IV). *L. plantarum* 3 had shown to be an efficient competitor in the fermented quinoa drink with potential for industrial use (Paper II and III).

5.1 Gut microbiota and probiotics

The gastrointestinal microbiota reaches a stable level and functional maturation around three years of age (Yatsunenko et al., 2012) and in adults, the majority of bacteria belong to the Firmicutes or Bacteroidetes phyla (Bäckhed et al., 2005). Despite the similarities between individuals on higher taxonomic levels, there is a high degree of variation in the gut microbiota composition on lower levels and within individuals since the bacterial community changes with age, health status, diet, and geographic location (Yatsunenko et al., 2012). The intestinal microbiota also changes in quantities and taxa composition along the gastrointestinal tract. The microbiota plays important roles in maintenance of intestinal integrity, competition with pathogens, immune functions, and in providing nutrients and sustain host energy balance by microbial biotransformation. However, the extent of the functions and the outcomes depend on the individual microbial composition.

The gut microbiota is fundamental for human health and there is increasing evidence linking imbalances in composition, so called dysbiosis, to diseases. However, it is still in many cases unclear if the disease develops due to a disrupted microbiota or if the microbiota changes as a response to the disease. Therefore, influencing the

gut microbiota by for example probiotic bacteria is an attractive way of disease modification or prevention.

Bacterial strains used as probiotics are frequently members of the genera *Lactobacillus* or *Bifidobacterium,* but even though species belonging to these genera are normal inhabitants of the gastrointestinal tract, they only constitute small proportions of the commensals (Arboleya et al., 2016; Walter, 2008).

Probiotic administration may shift the microbiota composition towards a more beneficial ecosystem and change its metabolic properties, which in turn may produce a different microenvironment due to a diverse range of metabolic pathway outcomes (O'Toole & Cooney, 2008). Even though colonization of probiotic strains in the gastrointestinal tract is a key factor to exert successful host-interactions, a resistance induced by the host microbiota may cause a transient nature for probiotic organisms. It is therefore likely that the microbiota returns to its original composition when probiotic administration is ceased. For natural reasons there are of course individual variations in the efficacy of probiotic colonization (Han et al., 2021).

One of the most known and well characterized probiotic bacteria is *L. plantarum* 299v, which is sold commercially and used as starter culture in Paper I. The strain has a proven ability to adhere to the gut mucosa by tannase binding and can compete with pathogenic bacteria. It has been shown to have effects against IBS symptoms and decrease intestinal permeability stimulated by *E. coli* (Mangell et al., 2002). However, probiotics with new mechanisms of action, which can be used for disease prevention and treatment, is in high demand. There are several characteristics that should be fulfilled by an ideal probiotic such as being non-toxic, non-allergenic and non-pathogenic, survive the passage through the gastrointestinal tract which involves resistance to low pH and bile salts, adhere to the intestinal cell wall, influence the immune system, and produce antimicrobial substances. In order to be attractive for the industry, it is also important for the probiotic bacteria to be resistant to production conditions, to survive and be able to multiply in large scale systems and to remain viable and in adequate amount during storage time.

5.2 Effects of *L. plantarum* 3 on the *Lactobacillus* group

In this thesis, one novel *L. plantarum* strain was used to investigate its ability to affect the microbiota. 20 volunteers were asked to drink the fermented quinoa-based beverage for two weeks, where *L. plantarum* 3 was used as starter culture, and saliva and faecal samples were collected before and after consumption. The participants were also asked to answer a short questionnaire to record experienced gastrointestinal well-being and digestive symptoms.

The results were analyzed using qPCR, terminal restriction fragment length polymorphism (T-RFLP) and NGS. Quantitative PCR is a method often used to determine the amount of a specific bacteria in a sample. The qPCR method used in this study was based on the SYBRgreen dye, which is a fluorescenting dye that attaches to double stranded DNA, but not the single stranded. The more DNA that is duplicated, the higher fluorescenting signal is detected. Together with a standard curve with known DNA concentrations, the amount of the bacteria in the sample can be detected. qPCR is one of the most common methods used when a quantitative result is wanted for bacterial analyzes. The results are highly dependent on the primers used for analysis, ensuring amplification of the correct fragment. In this study, primers previously evaluated (Karlsson et al., 2012) was used to determine the amount of *Lactobacillus* spp. in the samples.

In the trial, the amount of *Lactobacillus* species was found to increase over the study period, from a median of 8.28 log10copies/g to 9.71 log10copies/g in fecal samples (p<0.05), indicating alterations of the *Lactobacillus* group (Paper IV). Similar results have previously been found after consumption of probiotic products (Verdenelli et al., 2011). There was no significant difference between saliva samples taken before and after the consumption, implying constant levels of *Lactobacillus* spp.

5.3 Bacterial community changes induced by *L. plantarum* 3

The effect that a probiotic species has on the overall bacterial community is sometimes unclear. Compared to the already existing gut microbiota, addition of a probiotic species in relevant doses (10^6-10^9 CFU/ml) is a relatively small amount. Added to that, probiotics are often bacterial species belonging to *Lactobacillaceae* which is mostly active in the upper part of the small intestine, while the major bacterial community is found in colon. Previous studies have shown no effect on diversity measures after probiotic consumption in several diseases and conditions (Kaku et al., 2020; Quagliariello et al., 2016), while other studies indeed have found such a relationship.

T-RFLP is a method that can be used to study changes in bacterial communities. Usually, it is performed in order to calculate diversity indices such as Shannon's and Simpsons. T-RFLP is based on the principle that 16S rRNA genes cut with a restriction enzyme results in fragments of different length for different bacteria. Therefore, the number and amount of different fragments represent the bacterial diversity. Another, increasingly common method used for studying bacterial communities is NGS. In this thesis, NGS was performed using Illumina MiSeq

technology in order to get an overview of the bacterial composition before and after consumption of the probiotic drink.

Based on the results from the T-RFLP experiments, no change in Shannon nor Simpson diversity indices were found during the study. Shannon's diversity index was 2.37 (2.10-2.55) in saliva at the start of the study and ended at 2.31 (1.91-2.71) after consumption. In the fecal samples, the Shannon diversity index started at 2.63 (1.46-3.03) and ended at 2.67 (2.47-2.95). The same trend was seen for Simpson's diversity index. That there was no change in alpha diversity over the study is an indication that the overall bacterial community was not affected by the intake of the probiotic drink, but instead remained constant. The microbiota of adults is very stable (Yatsunenko et al., 2012) and the results from this study indicate that daily intake of the fermented quinoa beverage did not significantly shift the bacterial community structure. The same results were found after analysis with Illumina MiSeq. Changes were however found when studying relative abundance of amplicon sequence variants (ASV:s), especially among bacteria belonging to *Fusobacterium, Leptotrichia* and *Prevotella* in the saliva samples, which were seen to increase over the study period. In the fecal samples, bacteria belonging to *Bacteroides* seemed to increase over time. Those results indicate that while the probiotic bacteria did not influence the overall bacterial community, it did affect specific species both in saliva and fecal samples (Paper IV).

5.4 Key points

✓ The amount of *Lactobacillus* increased in the faecal samples while it remained unchanged in the saliva during the study period.

✓ No changes were found in alpha diversity after consumption of the fermented quinoa beverage.

✓ Specific bacteria were found to change in abundance over the study.

6. Future perspectives

The research performed in the present thesis is focusing on functional probiotic strains constituting a new concept of next generation probiotics, in which safe and known species expressing novel properties are used, instead of searching for species to date not approved as probiotics. The findings of certain properties expressed by the strains are of high significance for the dietary changes we are facing, when consumers are addressing their preferences for plant-based products as sustainable protein sources. Phenolic compounds are important constituents of plant-based foods, as their presence in low concentrations are related to protective effects on human health. However, chemical structures and interactions with complex carbohydrates are factors that hamper their bioavailability and digestion might be needed for them to exert their biological activity. Furthermore, in high doses phenolic compounds also have toxic properties and their antimicrobial effect can inhibit growth of intestinal commensals to levels that might be physiologically significant for development of disease.

The degree of biotransformation of specific dietary polyphenols in the gastrointestinal tract is determined by the individual richness of gut microbial species and genera, where some bioconversion can be performed by a vast array of microorganisms, meanwhile others require more specific chemical reactions and therefore also presence of species or strains expressing certain enzymatic capacities.

Among citizens in general, a higher intake of plant-based products will in all probability be the future dietary change. Achieving sustainable consumption is essential in development towards a more sustainable society, but the dietary change needs in turn to be followed by innovative and novel solutions for nutrient accessibility and prevention of antinutrients. The results obtained from the present work is therefore providing new candidates of the probiotic species, addressing new host targets. Adjacent to the probiotic market, in which there to date is no existing probiotic product specified for providing health benefits to consumers adapting to the dietary change occurring in our society, the innovation is also a development of a new variant of microbiome-modulating interventions, with renewed interest in polyphenols, fermentation and safety.

7. Acknowledgements

My gratitude to the Swedish International Development Cooperation Agency (SIDA), and to the International Science Programme (ISP) for funding the research. The PhD journey is a path where the planning most of the time will not work and where unexpected results can surprise you at the least expected time. But what is a common factor is people that are incredible, intelligent, strong, sweet, kind, happy and serious people will cross by this path while you are on it, transforming the experience in memories and unforgettable moments. Luckily for me, my journey started as part of the Food Hygiene group, the best research group with incredible people.

Siv Ahrné, my main supervisor during the first couple of years of my PhD, to you Siv my biggest gesture of gratitude for introducing me to the microbiology word and the trust that you put in my capacity. I really appreciate that you read the awfully badly written reports that I presented to you ☺ and that you made me feel "I am here to learn, and you will be there to guide me". To **Göran Molin**, thank you very much for the nice scientific discussions, support, suggestions, and short talks. I really appreciate that you invest your time reading the manuscripts, now published papers, which I am sure without your valuable comments would not have come with the same impact. To both Siv and Göran, thank you very much for keeping an eye on me and for sharing your knowledge.

To my co-supervisor (at that time), and who later became a main supervisor **Åsa Håkansson**, thank you so much for the support during all those years, not only on work related matters but also at a personal level 🌷. Thank you for your advice and for being present at the airport the day that my kids arrived in Sweden. It was the most kind and sweet gesture for them and for me.

To **Elisabeth Uhlig** for your kindness the first day I was on the lab. I was lucky and feel glad that you were there when all of this started. For your suggestions related to science, planning, positivity, calmness, and delicious-healthy snacks that you always would share 🌼.

The story behind the person who I shared office with during the years of my PhD, transforming the time to the nicest memories of talk, laughter, support with interesting scientifically discussions and who I am the proud to be able to say, "yes I know her" **Elin Oscarsson**, "yes, I shared an office with her" and "yes" she is very intelligent and kind. Dear Elin, the project would not have been the same without

you. I am very grateful for all the work, effort, and HUGE support that you have given, which the result of can be seen in this book. Thank you very much. 🌷

To **Anna Kjellström**, it was very nice for me to meet you during this time. The discussions would not have been the same without a cup of coffee (with scream or no scream during the summer) without you 🌻. Thank you very much for your help and valuable contributions to the project, and for reminding me that small details can also be specials.

My endless gratitude to **Margareta Sandahl** and **Marie Wahlgren** for accepting me as your student, thank you very much for all your invaluable contribution to the project.

To **Thamani Freedom** (the craziest analytical chemist, and hard worker), **Fiona Nermark** and **Daniel Padd**, thank you for the warm welcome to the world of analytical chemistry and for the support on my times of immense pressure. For your sincere friendship, for answering my basic questions regarding of HPLC, advice on analytical matters, observations, and nice constructive discussions.

To the co-authors, to whom without your effort the papers would not have been possible to be developed as greatly as they are now. **Tawanda Mandoga**, **Chandana Jois, Rownoke Jannat Janny, Jaison Sithole** and **Hanna Olsson**. Thank you all for allowing me to learn from you and for supporting me on my ideas, for the nicest meetings, from which I learned so much from each one of you. I have no doubt that you will shine in your lives and in your professional roles. I really hope that we can continue been friends and keep contact were ever in the world you are.

To my friends, and to whom I consider to be my first teachers who presented to me the world of microbiology as something interesting and complex to learn. Without your discussions, teaching, and explanations I would not have reached the level of where I am now. To **Afina Nuur Pharma Megaelectra**, **Gayatri Rajashekhar**, **Dooreen Jepngetich Metto**, thank you very much my friends 💐.

To the friends which made the everyday awesome in Sweden or in Bolivia, to **Vanesa Castro, Daniel Osorio**, **Atma Sol Bustos, Mukul Mahanti**, **Etzar Gomez**, **Nabilah Abdul Hadi**, **Stina Burri, Klara Sjölin, Yi Lu, Maria Mattos** (no one speaks faster than you Maria, but you have the biggest heart too), **Sumiyo Kanafusa**, **Grantt** and **Pui Thamkaek** (the sweetest couple I ever meet), and **Federico Gomez,** thank you all for the greatest moments.

A mis padres, **Juan Canaviri Balcazar (†)**, y **Rosario Paz de Canaviri** este esfuerzo no habria sido possible sin su ejemplo y apoyo, los amo. A mis hermanos **Alejandro, Vladimir y Zuleydi**, a y sus respectivas familias.

To my Swedish family, for opening your hearts and give me the opportunity to be part of this awesome family and for all the support.

To my kids, the real heroes here and the ones with the strongest and most sincere hearts **Tamara** and **Micaela**. I love you so much both 💗 and I am the luckiest mom.

To **Aleksander Sjörén**, well min sötnos no words can express how happy or how much grateful I am for be together. Jag älskar dig, min sötnos 💝.

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