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Review

Lactic acid bacteria as bioprotective agents: A mini-review on biotechnological-and human health-based facets

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Abstract: Despite the blitz on chemical-based fertilizers, the transition towards the bio-based fertilizers may subvert fossilfuels-based fertilizers. Research efforts are perpetrated to contend the transition towards sustainable agriculture, thus, enhancing the bio-based economy. Bioprotective agents are key players for alternative farming as environmentally-friendly applications. This mini-review provides a consolidated briefing of lactic acid bacteria production with the main focus on their impact on human health.

Keywords: LAB; fermentation, biofungicides; organic farming; mycotoxins, human health

1. Introduction

Plants are vulnerable to infections from pathogenic organisms, thus, a significant percentage of global food production may be lost due to these harmful activities [1]. Nowadays, the transition to alternative farming using natural or added microorganisms is crucial for the plants protection and production yield increase.

The use of biocontrol microbial agents (fermentates and their metabolites) to expand the food shelf-life and augment crops production is regarded as an efficient and sustainable biocontrol

technique. Biopreservation by using lactic acid bacteria (LAB) and their use in fermented foods has attracted interest in research efforts to replace current preservatives. Lactic acid bacteria (LAB) are Gram-positive and aerotolerant organisms which degrade carbohydrates via fermentation [2]. LAB can be phylogenetically divided into different genera. These genera include *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella* [2]. LAB have a symbiotic relationship with humans [3]. They are especially prominent in the gut.

Symbiotic relations between bacteria and plants can provide mutual benefits [4,5]. The earlier-mentioned relationship between humans and our gut bacteria is an example of organisms living in symbiosis. Humans provide bacteria with a protected environment, this includes protection against pathogens, temperature changes, stable pH, and a constant flow of nutrients. In return, these bacteria provide humans a stronger immunity against foreign pathogens and they help in breaking down nutrients [4,7]. This relationship has evolved over millions of years. As a result, the human body and all the bacteria living in it can be regarded as a superorganism [8]. The relationship between plants and bacteria in horticulture can easily be compared to this symbiotic relationship between humans and bacteria.

LAB in the gut can enhance a person's well-being. At this moment, extensive research is being conducted on the effect of probiotics on mood and decision making [7]. Bacteria in the gut, including LAB, are hypothesized to be able to communicate with the brain via several different signal molecules [9]. LAB are also very crucial in keeping the vagina healthy as they produce acids and hydrogen peroxide that can prevent yeast infections [10]. LAB have been used by humans for a long time in fermentation processes [11,12]. At this moment, the Netherlands is experiencing a nitrogen crisis [13]. This nitrogen crisis is not related to fungi, but it shows the inherent necessity to treat the soil with care. Not only do people employed in the sector see the need for innovation, but also interest from consumers in biological products has increased and is expected to increase more in the future [14]. The LAB described above can aid in biological protection. With a mixture of organic acids and other antifungal compounds, they can protect plants in horticulture against fungi and mycotoxins. This study aims to provide a brief overview of LAB production aspects as well as medical-related perspectives.

2. Current status of pesticides

Pesticides are chemicals, either natural or synthetic, that are used in agriculture to protect plants against diseases, and control pests. Pesticides include herbicides, insecticides, fungicides, and rodenticides [15]. The first reports about problematic pesticide use came from the sixties of the last century, most notably the famous book "silent spring" by Rachel Carson from 1962. This book kickstarted the environmental movement that was against the (excessive) use of chemical pesticides [16]. A study reported that 45% of all crops in the world are lost due to infestation [17]. The total amount of pesticides used in the world is more than 4 million tons [18]. The biggest pesticide user in the world is China with about 1.8 million tons [15]. The other major pesticide users in the world are the US and Argentina. Figure 1 shows that Asia has, with more than 50%, the highest use of pesticides of any continent in the world. China is mainly responsible for this high number as more than 80% of all pesticides in Asia are used in China. Other Asian countries with high

pesticide use are India and Japan. The Americas are responsible for 32% of all pesticide use. The major countries responsible for this are the US, Brazil, and Argentina. Europe occupies third place with 11.6% of worldwide pesticide use.

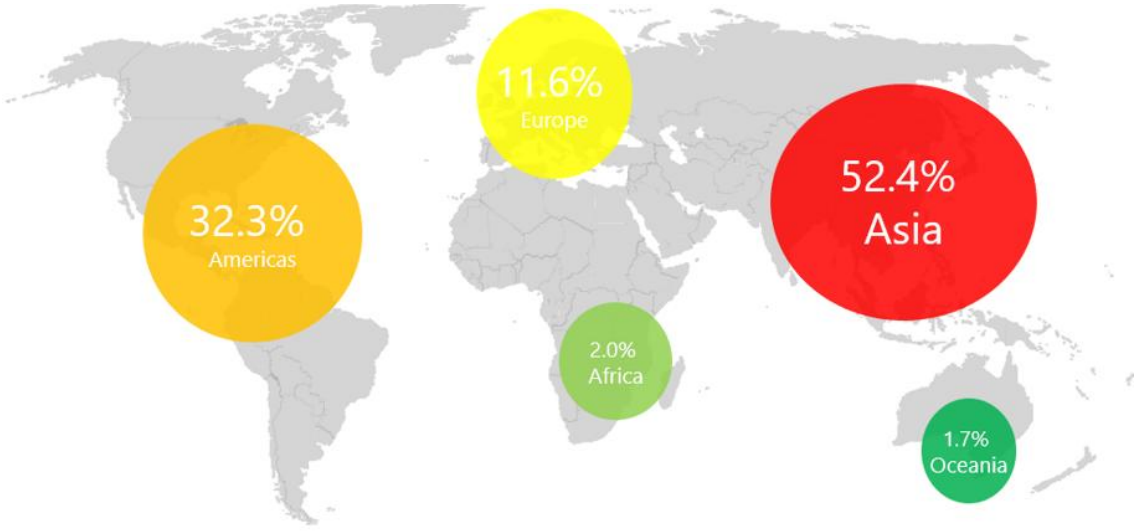

Figure 1. Total pesticide use per continent in 2018. Percentages are the share of the total worldwide pesticide use. Based on data from [18].

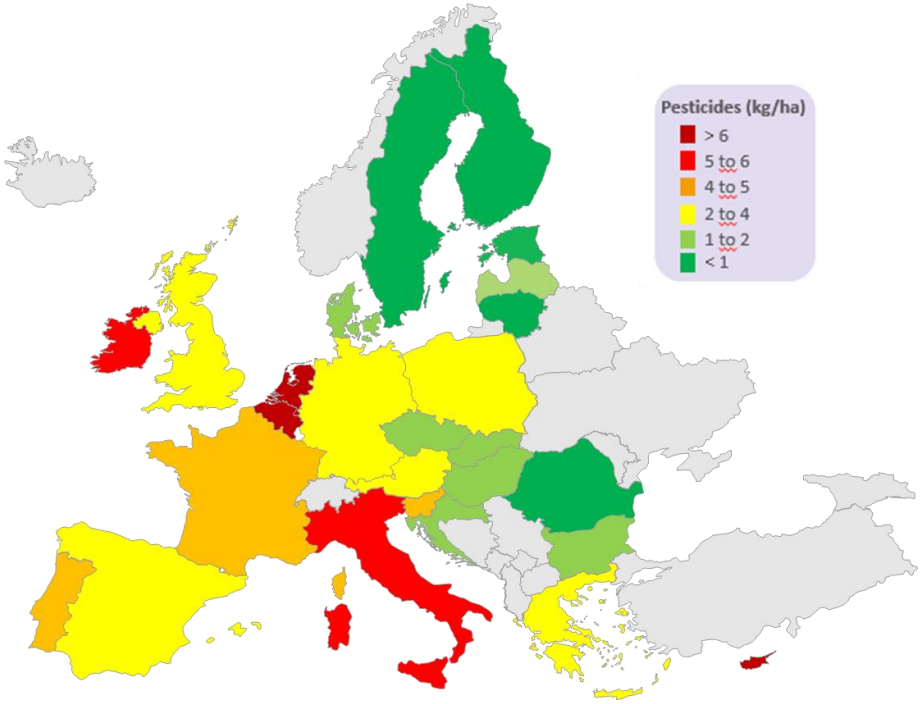

Figure 2. Pesticide use in (kg/ha) for each country in the EU-27 and the United Kingdom in 2018. Based on data from [18].

To check for toxicity and to make estimations on environmental impact, it is more interesting to look at the amount of pesticides used per hectare. Figure 2 shows the weight (in kg) of pesticides (or else density) used per hectare of cropland in Europe. Within Europe, the numbers are quite different

from country to country. The highest used is observed in Malta, Cyprus, the Netherlands, and Belgium. In absolute numbers, these countries do not use as many pesticides as bigger countries like France and Italy, but since they are smaller the environment will suffer more from the pesticide use. As can be seen in Figure 2, in general, western Europe uses more pesticides than eastern Europe. The lowest pesticide density can be found in northern Europe. The lowest pesticide use percentage-wise is in the continent Africa and Oceania. The explanation is the fact that Africa has less expertise than the rest of the world, also for several African countries, no data is available [18,19]. For Oceania, the explanation is that a relatively small part of the continent is suitable for crop production and it, therefore, has a lower crop production and pesticide use compared to other continents [20].

3. Transition to biopesticides: a human health-based perspective

Via their toxic properties, pesticides can have a negative impact on human health as pesticides can be metabolized, excreted, and stored in body fat [21]. A wide variety of people may be affected by the pesticides such as workers in agriculture, habitants in highly polluted areas, and consumers [22,23]. Since the LAB in this review can produce biofungicides, the main focus of this part of the review will lie on the negative effects of fungicides on human health. First, the most striking examples of the effects of pesticides on human health will be described. Additionally, a more detailed description will be given on the negative effects of fungicides. To conclude, the adverse effects on the human health of fungicides will be compared to the health effects caused by the use of biofungicides.

3.1. Negative effects of pesticides

Pesticides can harm human health in different ways. Notable ones are the development of Parkinson's disease and Alzheimer's disease [24–26]. Pesticides can also play a role in several cancers including bladder, prostate, and ovary cancer [26–29]. A study on the effects of different exposure levels of pesticides on greenhouse workers in China was performed [22]. This study tested the occurrence of a variety of adverse health effects that could be influenced by pesticides. The people were then divided into two groups, one group had experienced high exposure to pesticides and the other group had experienced low exposure to pesticides. The prevalence of diseases in the cardiovascular, skeletal muscle, digestive and respiratory systems showed an increase of 121%, 60%, 53%, and 47% respectively when the worker was exposed to a high concentration of pesticides. For the immune and endocrine systems, a correlation of −7% was found. Another study delved into the effect of organochlorine pesticides (OC) on the development of Parkinson's disease. The researchers examined at the concentration of some of the most common OC compounds in Parkinson's disease patients compared to a healthy control group. The results showed that the pesticide dichlorodiphenyldichloroethylene (DDE) was present in a higher concentration in patients suffering from Parkinson's disease [24].

After reviewing different papers, Kabir et al. (2018) concluded that chlorophenols that were contaminated with 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) increased the mortality rate of prostate cancer by 20% [26]. A study done by Silva et al. (2016) noted that many studies show that environmental and occupational exposure to endocrine-disrupting pesticides increases the risk of developing prostate cancer [30]. For women, pesticides can also impact the prevalence of ovarian

cancer. The pesticides responsible for this are endocrine-disrupting chemicals (EDCs) [30,31]. Pesticides that are EDCs include dichlorodiphenyltrichloroethane, methoxychlor, chlorpyrifos, and the fungicide vinclozolin. Apart from prostate and ovarian cancer EDCs can also induce other cancers, infertility, thyroid eruptions, nerve damage, and Alzheimer's disease [25,31]. The thyroid gland is another organ that can be badly affected by pesticides. The thyroid gland is responsible for the production of several hormones like T3 and T4 [33]. Exposure to pesticides can lead to abnormal changes in the concentration of these hormones. The researchers found that, in Spain, people that were highly exposed to pesticides were 49% and 41% more likely to develop the diseases hypothyroidism and thyrotoxicosis respectively [33]. A bigger factor in the risk of getting thyroid-related diseases is gender, with women 3 to 4 times more likely to get thyroid-related diseases. OC insecticides and chlorinated herbicides have been reported to cause hypothyroidism in female spouses of pesticide applicators [35]. Women are in general more susceptible to thyroid diseases and a correlation between pesticides and hypothyroidism for men was not found in this study.

3.2. Negative effects of fungicides

Here, the most severe negative effects of the most prevalent fungicides will be described. In the next paragraph, a comparison between the effect of these fungicides and the effect of bio-fungicides that can be used as an alternative will be made. Widespread azole compounds used in the growth of ornamental bulb plants are a major cause of global azole resistance patterns in e.g. *Aspergillus fumigatus* [36]. Fungicides may affect the thyroid of humans. A former study examined women living in farmhouses where pesticides are used and thyroid abnormalities appeared. Results showed that the fungicide maneb may influence the concentration of thyroid-stimulating hormone (TSH) critical for the functionality of the thyroid. The microbiome of the human gut and the microbial systems in the rhizosphere also show big similarities [37]. These communities share similar conditions in gradients of oxygen, pH, and water. The bacterial species inhabiting these communities are also similar, they enclose bacterial phyla of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* [38]. The communities also share a similarity in that they are both indispensable for the higher organisms with which they live in symbiosis. Humans need their microbiome to produce essential vitamins that the human body is unable to produce, additionally, the human microbiome protects the host against pathogenic bacteria [39,40]. Plants also need their microbiome in the rhizosphere, which is the area of soil close to the plants that is influenced by the plant. In this zone, the plant exudates nutrients allowing the bacteria living there to use them. In return, the microbiome can fixate nitrogen from the soil allowing the plant to use it. Additionally, the microbiome in the rhizosphere can modulate the immunity of the plants [41,42]. This system can, thus, be seen as an open-air gut. Researchers noted that, because humans eat plants, interactions between these two systems occur [37]. The use of pesticides lessens the richness of soils. This in turn can decrease the biodiversity of the human microbiome. The researchers note that these developments concur with an increase in diseases related to the human intestinal microbiome [37]. Utilization of biocontrol agents through bacteria can play a crucial role in plants' protection against pathogens as bacterial strains can enhance the resistance of plants against pathogens [43]. Biocontrol agents can replace chemical-based plant protection agents. Their use can play a significant role in human nutrition as organic, free of chemical pesticides, farming techniques can improve the quality of food [38].

Research studies showed that the toxicity level of biopesticides (including fungicides) is low in comparison to the toxicity level of regular pesticides [44]. Besides the benefits on human health, biopesticides and biofungicides use results in lower levels of water and soil contamination compared to chemical pesticides as lower amounts of organophosphate and carbamate pesticides are used [45].

A study has been done to compare the repercussion of bio-fungicides produced by *Bacillus subtilis* QST713 with a traditional chemical pesticide based on a fenhexamid chemical compound [46]. The fungicides were tested on the grape biofilm, must, and wine microbial diversity. The study found that biofungicides did not affect the species richness of the microbiota. The chemical fungicide, on the other hand, reduced the diversity of the microbiota in both the must and the wine. A similar study tested biological and chemical fungicides on Chenin blanc grapes [47]. The goal of the study was to find out if there would be any effect of the aroma, sensory, and chemical compounds. The authors concluded that both biological and chemical pesticides did not affect the wine. An older study found that bio-fungicides improved the yield and quality of tomatoes compared to tomatoes treated with a traditional fungicide [48]. The bio-fungicides were isolates of bacteria and fungi with known biocontrol against known fungal pathogens, for the chemical fungicide the researchers used Hymexazol.

3.3. Effect of the fusarium mycotoxins on human and animal health

The infection of plants by fungi infests farming activities and decreases the food production rate [49]. Additionally, plant infection by fungi results in non-edible food with significant health hazards due to produced mycotoxins from fungi [50]. Research showed the production of a wide variety of mycotoxins, i.e. trichothecenes, zearalenones, deoxynivalenol, T-2 and HT-2 toxins, beauvercins, enniatins, fumonisins, and fusarins produced by the fungus *Fusarium*, aflatoxins produced by *Aspergillus* species, and ochratoxin A by *Penicillium* and *Aspergillus* species [49,51,52].

An analysis performed by the FAO, states that the preponderance of mycotoxins in grain is up to 60% of deoxynivalenol and up to 80% of zearalenones [52]. This study states that climate change will worsen food contamination by mycotoxins. Although mycotoxins are regarded as unnecessary for the fungal metabolism, mycotoxins are used as secondary metabolites focusing on self-defense mechanism [53]. Environmental conditions, i.e. changes in temperature or moisture, provoke mycotoxins generation but have no repercussions on the growth of fungi [54]. The presence of antifungal substances can trigger the production of mycotoxins as fungi develop coping mechanisms and encounter higher stress when they adjoin with these compounds [53]. LAB can be used as a protecting tool to detoxify mycotoxins through degradation or mycotoxin binding. *F. fungus* has been previously studied regarding the pathogenic interactions in agriculture and its impact on crops in diverse climate zones [55]. A former study states that *F. graminearum* and *F. oxysporum* strains are considered important fungi in molecular plant pathology [56]. *Fusarium* fungi strains are of high importance and contribute to the negative effects of mycotoxins on humans.

However, in modern agriculture intake of dosages that have severe effects are extremely rare. Therefore, it is more interesting to look at the effects of more moderate dosages [57]. After oral intake of mycotoxin, the first cell that will come into contact with the mycotoxins is an epithelial cell in the gastrointestinal tract. Here they affect the cells present in the gut. For goblet cells, cells that produce mucus that cover the intestinal tract, a combination of deoxynivalenol, zearalenones and T-2 causes a decreased amount of mucus production in pigs, while exposure to only zearalenones will

cause an increase in mucus production. the mucus produced by goblet cells functions as a protective layer over epithelial cells. Changes in mucus production will therefore inhibit the protective effects provided by the goblet cells. Deoxynivalenol, zearalenones T-2, and FB1 can all alter cytokine production, additionally, deoxynivalenol and T-2 affect immunoglobulin A [57,58]. The intestinal permeability is increased by and FB1, this causes the entire body to be more exposed to pathogens and it also reduces the selectivity in which nutrients can be taken up by the body.

Former studies indicated the impact on adaptive immunity by *Fusarium* mycotoxins, as they impede the production of the antigens, influence the generation of macrophages and neutrophils and weaken the activity of t-cells and b-cells [59,60]. The impact of *Fusarium* mycotoxins on the human body is directly connected with the immune system as mycotoxin enhances pathogens' capability to infect the host [57]. Significant research has also been performed on the bacterium *Salmonella* that can cause gastroenteritis in humans [61,62]. Another study investigated the diseases caused by mycotoxins in pigs due to similarities of the metabolism and internal organs to humans [63]. In pigs, deoxynivalenol and T-2 mycotoxins can assist in the transepithelial passage of salmonella. Then, Salmonella will be taken up by the host's macrophages. Deoxynivalenol and T-2 can enhance the uptake of salmonella by modulating the cytoskeleton of macrophages, increasing the ability of macrophages to engulf *Salmonella*. Literature shows, that a moderate to high amount of T-2 toxin in combination with *Salmonella* increases mortality among mice [64,65].

4. Indicative metabolites produced by LAB

4.1. Organic acids

The most obvious of these acids is in the name of the bacteria, lactic acid. Lactic acid is not the only acid produced by these bacteria, but acetic acid, propionic acid, caproic acid, and phenyllactic acid (PLA) are also enclosed. Acids cause a problem for the fungal membrane [66]. Undissociated acids can pass the cellular membrane as they are lipophilic. The increase in the pH causes the acid molecules to dissociate, this leaves the protons stuck inside the fungal cell [67]. This decreases the pH in the cytosol. The buildup of the protons inside the cellular membrane disrupts the proton motive force inside the cells. Thus, the buildup of acids in the cytoplasmic membrane halts glycolysis [68,69]. Halting the production of ATP. The disruption of ATP production will slow down the metabolism within a cell and thereby stop cell growth.

The process of dissociation of acids happens at different pKa for each acid. A higher pKa will mean that the acids dissociate at a higher pH, making it a more effective antifungal compound [54]. In a previous study, researchers stated that a low concentration of lactic acid is less effective in inhibiting fungal growth than acetic acid [70]. To test whether these acids were the only things responsible for the inhibition of fungal growth, the researchers added proteinase K to the supernatant of the bacteria. This enzyme will break down proteinaceous compounds that can also affect fungal growth. A previous study showed the interrelation of the antifungal activity of 8 LAB species and their growth medium. A previous study examined the impact of growth medium on the antifungal activity of LAB. Specifically, researchers examined the growth of *L. plantarum, L. paracasei*, *L. fermentum*, and *L. brevis* in different media. They noticed that the addition of phenyl pyruvic acid (PPA) enhanced the antifungal activity. They correlated this enhancement with the production of phenyllactic acid (PLA). They also reported the presence of a PPA derivative named polyporic acid

that may increase antifungal activity. In this study, the researchers also identified a strong negative correlation between the pH and the antifungal activity. Previous studies reported that hydroxy- and phenyllactic acids have antifungal activity and, thus, avert the rapid spoilage in feed silage, curated meats and malting of barley [72–74].

4.2. Reuterin

Reuterin is a growth inhibitor, produced by Gram-positive bacteria. It has significant effectiveness against several fungi such as *Fusarium spp.*, *Penicillium spp.* and *Aspergillus spp.* [75,76]. A study found that reuterin causes oxidative stress in *E. coli* [77]. The authors found that many genes involved in the oxidative stress response were overexpressed when the bacteria were exposed to reuterin. Additionally, they found that *E. coli* defective in the OxyR-mediated oxidative stress response was more sensitive to reuterin. In a previous study *L. reuteri* ATCC 53,608 was used to produce reuterin [76]. The researchers examined the impact of reuterin against the fungi *P. chrysogenum* LMA-212 and *M. racemosus* LMA-722 in yogurt. The authors stated that fungal growth was enhanced when a concentration lower than 5 mM was applied during incubation for a period of 21 days. At 1.38 mM the fungal growth was inhibited by 3 days compared to the positive control. With fungal growth visible on the 7th day for the positive control and the 11th day for the fungi that grew in the presence of 1.38 mM reuterin. The researchers further noted that although *L. reuteri* has been classified as safe by the European Food Safety Authority (EFSA), only limited research has been done on reuterin regarding toxicokinetics and long-term exposure [78].

In a former study, authors investigated the synergistic reverberation of reuterin with PLA. The authors discovered that reuterin is very reactive at higher temperatures, making it ineffective as a food preservative when the food is heated [79]. The high reactivity of reuterin is also the reason no synergetic effects between reuterin and PLA could be detected. Reuterin reacts with the free amino group in phenylalanine, the precursor of PLA. This reaction completely nullified all antifungal activity.

4.3. Fatty acids

Fatty acids have been considered as antifungal compounds by the disintegration of the plasma membrane [80–82]. In a former study, authors examined the impact of the sterol concentration present in the fungal membrane in the effectiveness of the fatty acid cis-9-Heptadecenoic acid (CHDA) [80]. They stated that high sterol content increases the resistance of fungi *I. bolleyi* and *P. rugulosa* to CHDA. On the contrary, *P. infestans* and *P. aphanidermatum* showed low resistance to CHDA as they contain no sterol [82].

Another study found that fatty acids of a certain size are more effective as an antifungal agent than other sizes of fatty acids [81]. Fatty acids with a tail of 10 (capric acid) and 12 (lauric acid) carbon atoms showed the highest antifungal activity. The total inhibition by these fatty acids amounted to more than 75% for capric acid and about 60% for lauric acid against the fungus *Candida albicans* at a concentration of 10 mM. Another study showed that 4 3-hydroxy fatty acids produced by *L. plantarum* MiLAB 14 can reduce fungal growth [82]. The MIC values of these compounds have been determined at 10-100 μg/ml. They also reported that synergetic effects between the different fatty acids seem to occur. Coriolic acid supplemented bread showed an increase

in the bread shelf life of bread inoculated with *P. roqueforti*, *A. niger*, and environmental contaminants with 7, 2, and 8 days, respectively [84]. Indicating the effectiveness of coriolic acid and the octadecenoic acid produced by *L. hammesii*.

5. Production of LAB

5.1. MRS and M17 media for LAB growth

The best-known and used medium for the growth of *Lactobacillus* are de Man Rogosa and Sharpe (MRS) medium [85–87]. This medium was specially developed for the growth and development of Lactobacillus. Another well-known medium is the M17 medium. This medium was developed for *Streptococcus* [87]. First, a closer look will be taken at the compositions of these standard media for LAB (Table 1, Table 2).

Table 1. Composition of the commercially most common MRS media [86].

	Composition of MRS broth for 1 liter of medium (g/L)				
Component	MRS (original)	Merck	Neogen	Sigma	Difco
Peptone	10.0	10.0	10.0	10.0	10.0
Beef extract	10.0	8.0	10.0	10.0	10.0
Yeast extract	5.0	4.0	5.0	5.0	4.0
Dextrose (Glucose)	20.0	20.0	20.0	20.0	20.0
Sodium acetate CH3COONa	5.0	5.0	5.0	5.0	5.0
Tween 80	1.0mL	1.0mL	1.0mL	1.0mL	1.0mL
Disodium phosphate Na2HPO4	2.0	0.0	2.0	2.0	2.0
Ammoniun citrate NH4C6H5O7	2.0	2.0	2.0	2.0	2.0
Magnesium sulfate MgSO4.7H2O	0.2	0.2	0.1	0.1	0.2
Manganese sulfate MnSO4.5H2O	0.05	0.04	0.05	0.05	0.05
Dipotassium phosphate K2HPO4	2.0	2.0	0.0	0.0	0.0
Final pH at 25°C	6.2 – 6.6	5.7 ± 0.2	6.5 ± 0.2	6.5 ± 0.2	6.5 ± 0.2

In the medium, peptone is a protein source. Peptone contains proteins and amino acids that can be used as an N-source. The beef extract is made from the bone, muscle, or skin of a cow. Using hydrolysis, the animal products have been reduced to single amino acids or peptides. Glucose (dextrose) is a simple sugar molecule that can be used for glycolysis, which is essential for energy production and growth in a cell. Sodium acetate is necessary for its buffering potential [86]. Tween 80 aids cells in the uptake of nutrients by increasing the permeability of the cell membrane [89]. Manganese (Mn^{2+}) and Magnesium (Mg^{2+}) are essential for metabolic activity in a cell [90]. Sodium (Na^{+}) and Potassium (K^{+}), together with Mn^{2+} and Mg^{2+}, are required by most LAB for nutrient transport and enzymatic activity.

Different LAB can prefer slightly different media [91]. A former study showed that *L. rhamnosus* R-2002 showed 20% more growth on 10 g/L formic acid and 10 g/L glucose than on the control of 20 g/L glucose [92]. Additionally, this study shows that bacteria exhibit more antifungal activity when they are grown on media containing glycerol and ethanol. The authors claimed that this is caused by a synergetic effect between the formed end products and ethanol. The addition of certain

components to a medium can also enhance the production of the desired secondary metabolites, as described earlier.

Table 2. Composition of the commercially most common M17 media [86].

	Composition of M17 broth for 1 liter of medium (g/L)			
Component	M17 (original)	Oxiod	Sigma-Aldrich	Merck
Tryptone peptone	5.0	5.0	2.5	2.5
Polypepton	5.0	0.0	0.0	0.0
Soya peptone	0.0	5.0	5.0	5.0
Beef extract	5.0	5.0	5.0	5.0
Yeast extract	2.5	2.5	2.5	2.5
Ascorbic acid	0.5	0.5	0.5	0.5
Magnesium sulphate	1.0	0.25	0.25	0.25
Di-sodium-glycerophosphate	19.0	19.0	19.0	19.0
Lactose	5.0	0.0	5.0	5.0
Meat peptone	0.0	0.0	2.5	2.5
pH (at 22 – 25°C)	7.15 ± 0.05	6.9 ± 0.2	7.0 ± 0.2	7.2 ± 0.2

5.2. Fermentation process for LAB production

Fermentation is a process in which organic nutrients are being degraded aerobically or anaerobically. Fermentation has been widely used to produce pivotal compounds for food and has been used in the chemical and pharmaceutical industries for years [93]. Bacteria can produce a variety of secondary metabolites. Examples of secondary metabolites include bacteriocins, pyrazines, and indole [94]. The quantity of these secondary metabolites can, however, be exceptionally low. In a fermentation process we, thus, want to optimize all factors to enhance the production of the desired end products. The optimization of the process entails optimizing the growth conditions. As explained before, this entails having the right resources, conditions and incubation time [95]. There are three main types of fermentation. These types are batch, fed-batch, and continuous. In batch fermentation, all substances are present from the start of bacterial growth. This means that during the fermentation process itself, no compounds, neither new growth substrates or end product, will enter or leave the fermentation process (e.g. bioreactor). A batch fermentation is the easiest kind of fermentation that, therefore, requires the least amount of control [96]. In a fed-batch, the culture is constantly fed without the removal of the end-product. The bacteria are usually fed with a C-source like glucose [97]. In continuous fermentation, the culture is mixed and operates in steady-state conditions. This steady state is achieved by consistent feeding while an equal volume of the converted substrate containing both the product and the producer is removed [98].

A recent study tried to find out which of these 3 methods would give the highest yield of lactic acid produced by *L. casei* [99]. For the batch culture, 20–70 g/L glucose is used. For the fed-batch feeding happened in 3 different ways: pulse feeding, constant feeding, and linear feeding. In pulse feeding, 9.5 grams of glucose was given in intervals of 4 hrs. In continuous feeding each hour, 3.5 grams of glucose was added. In linear feeding, glucose was added according to a formula that included biomass concentration and the percentage of substrate consumed (Figure 3). In the continuous method, cells were either free or immobilized in a bioreactor. The researchers found that

fed-batch pulse feeding yields the highest concentration of lactic acid after fermentation, while the productivity (in g/h/r) was highest for 60 g/L glucose in the batch at 4.02 g/L/h, for continuous fed-batch at 4.01 g/L/h and 4.30 for the immobilized continuous culture.

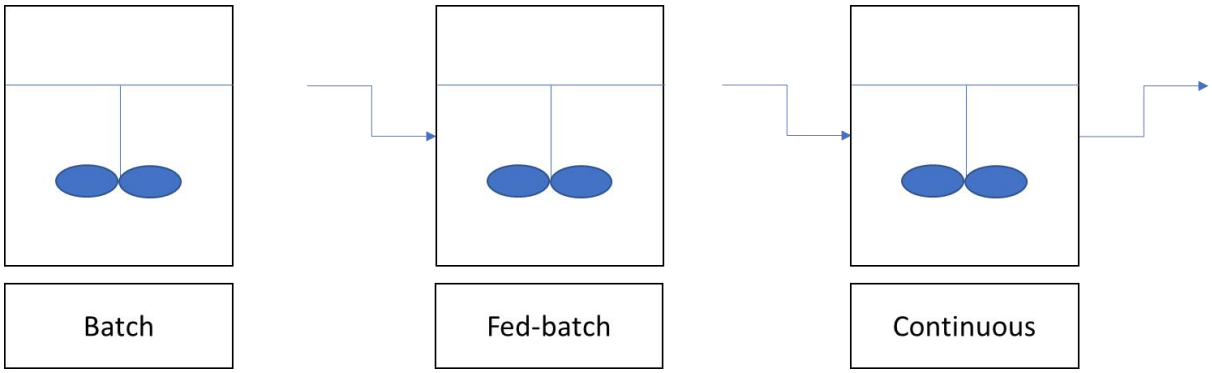

Figure 3. Schematic representation of the fermentation processes. Batch feeding: all substrates and producers are present from the start and nothing will enter or leave the fermentation process. Fed batch: culture is constantly fed with new substrate. Continuous: culture is constantly fed while and equal volume of end-product and producers are constantly removed.

Another study looked at the differences between a homofermentative LAB (*L. sakei*) and a heterofermentative Lactic acid bacteria (*Lc. mesenteroides*) in the fermentation of kimchi [100]. The researchers found that hetero fermentation created more mannitol and acetic acid, but less lactic acid was produced. *Lc. mesenteroides* acidified the environment faster, which means that this strain grows faster and therefore produces secondary metabolites earlier in the fermentation process. A mixed culture could be very useful for the final formulation of antifungal compounds as different acids can exhibit synergetic effects. The authors made several cultures with 0/25/50/75/100% of *Lc. mesenteroides* and *L. sakei*. The authors found that in the first 24 hours of the fermentation the *Lc. mesenteroides* was dominant, as more acids were produced by the *Lc. Mesenteroides* strain and therefore the pH reduced faster than the medium dominated by *L. sakei*. After 72 hours *L. sakei* produced more acids, which means that this strain is more dominant later in the fermentation process. The authors believe that a mixed culture is the best for the fermentation of kimchi as the LAB can compensate for each other's weaknesses.

LAB often suffers from end-product inhibition [101]. As lactic acid bacteria produce organic acids which can inhibit the growth of LAB the same way it inhibits the growth of another microorganism. The conventional way to overcome this end-product inhibition is by the addition of bases like calcium hydroxide. The dissolved calcium must then be filtered by sulphuric acid, as it will precipitate as gypsum. The process costs a lot of sulphuric acids, however, and generates large amounts of waste [102]. Another way to keep the concentration of organic acids below toxic values would be by using fed-batch fermentation. In fed-batch fermentation, the production of organic acid can be optimized so that it never reaches toxic concentration using optimized intermittent feeding. Additionally, alkaline molecules can be added to keep the organic acids below toxic levels [103]. A problem that arises here is that the constant addition of NaOH will increase the osmotic pressure which also inhibits LAB growth. The researchers concluded that when an osmotic value of 2416 $mOsm*kg^{-1}$ was reached, the growth of *L. plantarum* was inhibited completely [103]. A review by

Othman et al. (2017) identifies 4 fermentation extraction approaches that overcome end-product inhibition [101]. These approaches are solvent extraction, electrodialysis, aqueous two-phase systems, and adsorption.

5.3. Extraction process for LAB recovery

In the solvent extraction process, lactic acid will first be extracted from the culture by an extractant, after which lactic acid can be recovered using back extraction into another solvent. Because lactic acid is quite hydrophilic, it can be hard to separate using common organic solvents. A study by Gao et al. (2009) tried to extract lactic acid from a culture containing the genetically engineered yeast strain *Saccharomyces cerevisiae* OC-2T T165R [104]. They used tri-n-decyl amine (TDA) as an extractant. Because of its long aliphatic chain, TDA has a low solubility which makes the separation of solvent and the culture easy. The researchers found that while TDA, which contained impurities of which 1-decyl aldehyde is known as toxic to yeast. When they reduced the amount of 1-decyl aldehyde from 700 ppm to 33 ppm yeast growth was no longer inhibited and productivity of lactic acid had improved compared to the control without added solvent. In electrodialysis, an ion exchange membrane is used for in situ removal of lactic acid. In this method, electric fields will be used as a driving force to remove ions present in the culture. The main application of this method is to concentrate ionic substances and to remove salts and waste generated from the processes.

A study by Habova et al. (2001) used electrodialysis to increase the concentration of lactic acid produced by *L. plantarum* L10 by 2.5 times by desalting it with electrodialysis using ion-exchange membranes [105]. In the second step of the electrodialysis, the concentration of lactic acid was further increased from 111g/L to 157g/L using the electro-conversion of sodium lactate to lactic acid. The main disadvantages of electrodialysis are high operation costs and deionization of the culture broth [101,106]. In aqueous two-phase systems (ATPS) biomolecules are partitioned using two liquid phases formed by mixing polymer and salt or two polymers and water [107]. ATPS can also be used for lactic acid removal. It will, then, consist of polyelectrolyte, poly(ethyleneimine) (PEI), and the neutral polymer hydroxyethylcellulose (HEC) [108]. Nowadays, the usefulness of ATPS is limited by lower effectiveness and a higher price [101]. Adsorption can be used in extractive fermentation. Adsorption can be used to improve LAB fermentation suffering from either product or by-product inhibition. Adsorption can be described as an accumulation of a gas or liquid onto a molecular or atomic film. These films are often made up of activated carbon, molecular sieves, and some other low-cost materials. In adsorption, sorption isotherms can be used to describe the equilibrium relation between adsorbate and adsorbent, this then gives the capacity of an adsorbent to adsorb the absorbate. A previous study by Gao et al. (2011) used extractive fermentation with activated carbon in a pH uncontrolled environment [104]. In this study, the inhibitory effect of lactic acid was diminished which enhanced both the growth and productivity of the genetically engineered *Saccharomyces cerevisiae* OC-2T T165R strain.

6. Conclusions

Biofungicides, like the ones produced by LAB, have shown lower toxicity for animals, including human. Numerous experiments have shown the effectiveness of these biofungicides.

Additionally, the natural symbiotic relation between LAB and plants can enhance the plants' immunity. Also, a study showed that the natural fungicides and LAB improve the quality of the final product compared to traditional fungicides. These features make LAB and their products key candidates to replace harmful chemical fungicides contributing to a more sustainable way of agriculture.

Conflict of interest

We declare no conflict of interest.

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