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REVIEW ARTICLE

Adaptive Laboratory Evolution to obtain lactic acid bacteria strains of industrial interest – a review

Adaptação Laboratorial Evolutiva para obtenção de cepas de bactérias ácido-lácticas de interesse industrial – uma revisão

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Abstract

The purpose of this review was to describe how adaptive laboratory evolution (ALE) can provide improvement to lactic acid bacteria (LAB) strains for their application in industrial biotechnological processes. This review was carried out according to the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) approach, incorporating the ScienceDirect and Scopus databases. The literature search yielded 4,167 (ScienceDirect) and 27 (Scopus) articles, which were reduced to 12 after applying the inclusion /exclusion criteria. The studies revolved around LAB of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Enterococcus* and the application of ALE experiments in batch mode, fed-batch mode, or both, and aimed to produce strains with increased lactic acid production capabilities, higher cell viability, and multiple-stress tolerance. The studies demonstrated that ALE is an efficient approach for strain modification towards desired phenotypic functions and does not require genetic engineering. Knowledge of the cellular and molecular responses of microorganisms to stress enables an understanding of the adaptation mechanisms of LAB strains for survival and increased production of metabolites throughout ALE experiments.

Keywords: Enhancement; Evolution; Metabolism; *Lactobacillus*; *Lactococcus*; *Leuconostoc*.

Resumo

O objetivo deste artigo de revisão foi descrever como a adaptação laboratorial evolutiva (ALE) pode proporcionar o melhoramento de cepas de bactérias ácido-lácticas (BAL) para aplicação em processos biotecnológicos industriais. A revisão foi realizada de acordo com a abordagem *Preferred Reporting Items for Systematic Review and Meta-analysis* (PRISMA), empregando as bases de dados ScienceDirect e Scopus. A busca bibliográfica resultou em 4.167 e 27 documentos, respectivamente, os quais reduziram-se para 12 após a aplicação dos critérios de inclusão/exclusão. Os trabalhos utilizaram BAL dos gêneros *Lactobacillus*, *Lactococcus*, *Leuconostoc* e *Enterococcus* para a ALE, empregando o método batelada ou batelada alimentada ou ambos, e tinham como objetivo a geração de cepas com maior capacidade de produção de ácido láctico, viabilidade celular e tolerância a diferentes condições

de estresse. Os estudos demonstraram que a ALE é uma ferramenta eficiente no melhoramento de cepas para os fenótipos desejados, além de não empregar engenharia genética. O conhecimento das respostas celulares e moleculares dos microrganismos ao estresse possibilita a compreensão dos mecanismos de adaptação das cepas de BAL para sobrevivência e maior produção de metabólitos ao longo da ALE.

Palavras-chave: Melhoramento; Evolução; Metabolismo; *Lactobacillus*; *Lactococcus*; *Leuconostoc*.

Highlights

- Adaptive Laboratory Evolution (ALE) increases the possibilities for food industry
- ALE can improve the production of metabolites by lactic acid bacteria
- ALE major challenge is to introduce the evolved strains in real food products

1 Introduction

Lactic acid bacteria (LAB) are microorganisms employed in the production of fermented foods. LAB may act as *starter cultures*, generating lactic acid (LA) as the main product of the fermentation of sugars, or as *non-starter cultures*, mainly producing aromatic compounds (Papadimitriou et al., 2016). Fermentation processes are used to preserve and improve sensory characteristics, such as the aroma and texture of different foods (Gadaga et al., 2007; Fuka et al., 2013; Kostelac et al., 2020). LAB strains are selected according to the desired phenotypes so that they are able to impart particular characteristics to these products (Johansen, 2017).

Recombinant DNA technology is one of the techniques used in the manipulation of genetic material to obtain LAB with desired phenotypes. However, the use of this technology has restricted applications within food industries that use LAB cultures for the production of fermented foods, mostly due to regulatory concerns and a perceived lack of consumer acceptance (Džidić et al., 2003; Derkx et al., 2014; Johansen, 2017). Therefore, the food industry mainly opts to use methods that generate spontaneous mutations to obtain LAB strains with phenotypic functions of interest. Within this context, Adaptive Laboratory Evolution (ALE) is a promising biotechnological tool and experimental approach for the development of industrially viable strains, aimed at improving the survivability of LAB under adverse conditions and the production of metabolites of interest, without having to employ a genetic engineering method (Drake & Mckillip, 2000) such as recombinant DNA.

This method imitates natural evolutionary processes that consist of repeating cycles of genetic diversification. Therefore, the adapted strain is selected from a genetically diverse pool efficiently generated via natural or induced mutagenesis (Sandberg et al., 2019). ALE experiments allow the development of LAB strains with characteristics such as a higher tolerance towards multiple types of stress or the production of particular metabolites of interest, such as lactic acid and aromatic compounds. Strains that show potential in order to act as probiotics can also be produced. In short, ALE allows the production of microbial strains for the production of fermented foods with unique sensory characteristics and/or that provide significant health benefits to consumers (Steele et al., 2013; Rakhmanova et al., 2018). Several studies have been performed using this method to improve industrial LAB strains, such as increased lactic acid production (Zhang et al., 2012; Chen et al., 2015; Ju et al., 2016; Mladenović et al., 2019; Liang et al., 2020), thermotolerance (Chen et al., 2015; Kwon et al., 2018; Liang et al., 2021), multiple-stress tolerance (Ming et al., 2016), increased vitamin production (Liu et al., 2021) and the production of aromatic compounds of industrial interest (Liang et al., 2021).

Considering the biotechnological significance of enhancing LAB traits without the need for specific genetic engineering techniques, this review aims to describe how ALE can provide the desired improvements to LAB strains for their application in bioprocesses within the food industry. Thus, this review addresses and discusses recent studies that have developed acid-tolerant and thermotolerant LAB strains, resulting in increased lactic acid production and better microbial viability. In addition, the results are presented in a table that provides readers with

information about the research objectives, the identification of the LAB used, the adaptive conditions employed, and the main results that were achieved. This review intends to provide new insights from scientific and practical perspectives regarding the application of ALE in food biotechnology processes that depend on LAB.

2 Materials and methods

The Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) methodology (Salameh et al., 2020) was used to structure this review. A bibliographic search was performed in the ScienceDirect and Scopus databases, using the keywords "*lactic acid bacteria*" and "*adaptive laboratory evolution*". The inclusion and exclusion criteria were: a) year of publication – only articles published between 2012 and 2022 were included; b) type of document – only research articles were included; c) language – only articles written/published in English were included; d) Impact Factor (IF) lower than 2.0 were excluded; e) short communications and scientific opinion articles were excluded; and f) literature reviews were excluded. Article selection began with the reading of titles and abstracts, and only those that showed results related to the topic of ALE of LAB were included. After selection, the articles were read in full and the data was organized by the morphology of the microorganisms (bacilli and cocci). The results obtained in the studies were presented in tabular form.

3 Results and discussion

The search in the ScienceDirect and Scopus databases resulted in a total of 155 documents after refinement (by year of publication, type of document, and title). This number was reduced to 12 articles after the application of the inclusion and exclusion criteria. Figure 1 details the selection process of this systematic literature review.

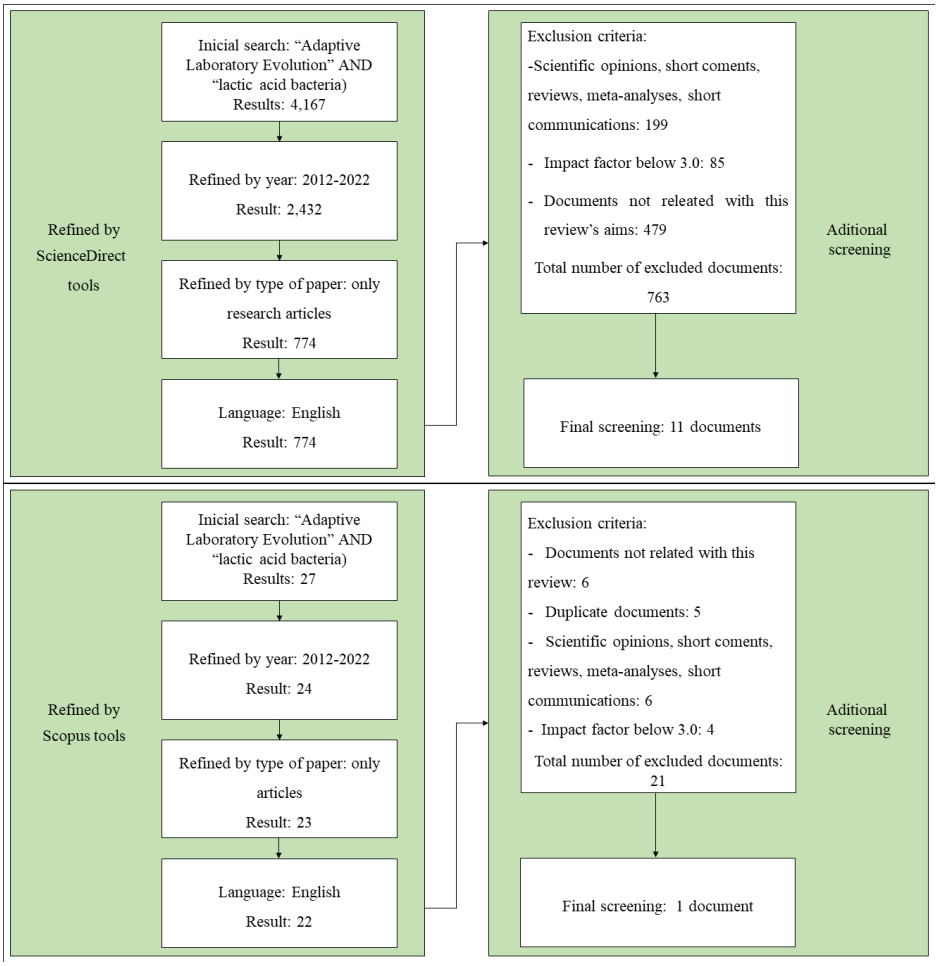

Figure 1. Study selection process, including search queries and inclusion criteria.

Among the 12 selected articles, ALE was used for the improvement of two LAB genera (*Lactobacillus* and *Lactococcus*). Studies were found to focus on nine strains of *Lactobacillus* and three strains of *Lactococcus*. ALE was used as a tool to mainly improve or adapt LAB toward an increase in lactic acid production capacity, an increase in survival rate at acidic pH values, a better tolerance to different temperature conditions, a higher vitamin production, the maintenance of genetic stability of the strains under multiple stresses, and the investigation of how antibiotics can result in adaptations within the LAB genome. Among the different ways that ALE can be applied (Figure 2), it was observed that, of the 12 studies cited, seven utilized batch mode ALE (Zhang et al., 2012; Chen et al., 2015; Ming et al., 2016; Kwon et al., 2018; Prasad et al., 2020; Liang et al., 2021; Liu et al., 2021), one utilized the fed-batch mode method (Singhvi et al., 2018), and one utilized batch, fed-batch, and pulse fed-batch modes (Mladenović et al., 2019). The remaining works used distinct methods that are not described in Figure 2.

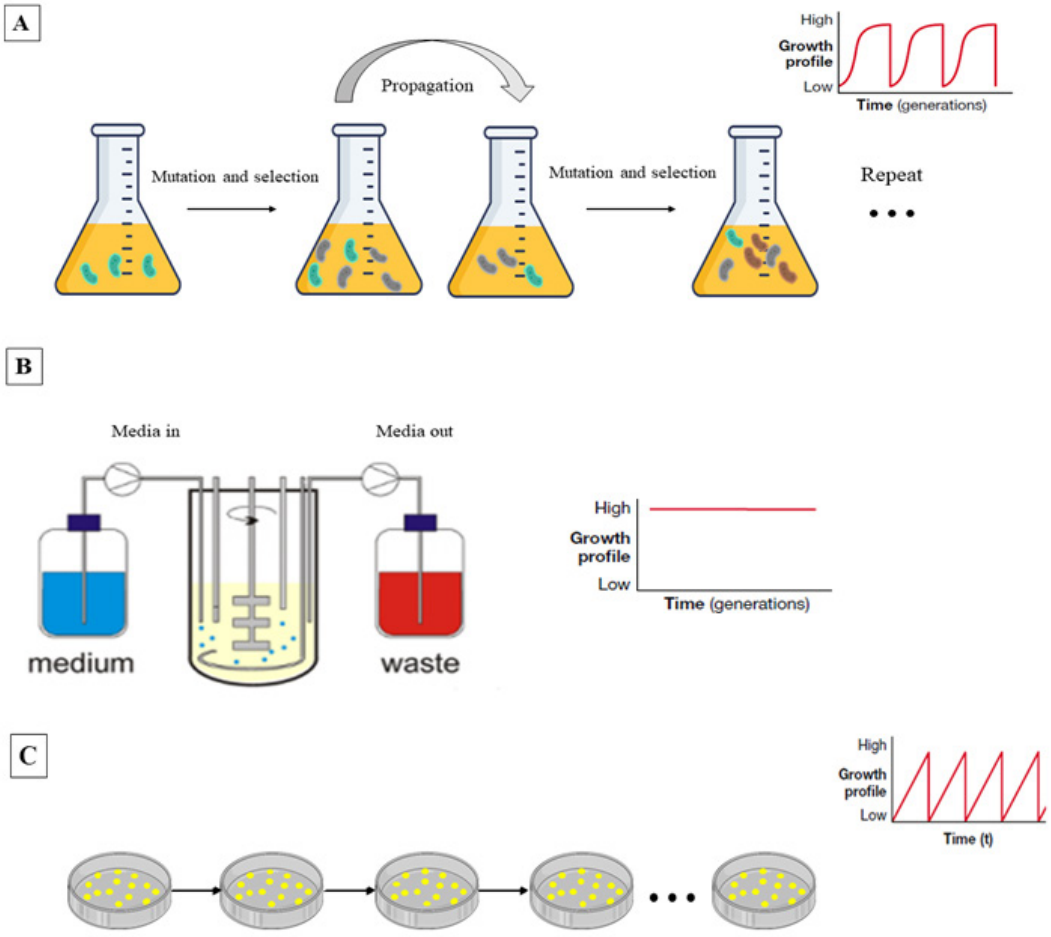

Figure 2. Methodologies used in ALE experiments: (A) Serial dilution (batch mode or fed-batch mode): this method imitates the natural evolutionary processes, in which the modified strain is obtained from a genetically diverse pool produced via (natural or induced) mutagenesis. To this end, aliquots of a culture are transferred into a fresh culture medium at regular intervals, and the growth of a microbial population is related to the generation time of each microorganism. (B) Chemostat: microbial cultures are kept in bioreactors with a continuous supply of fresh culture medium, resulting in constant microbial population growth rates and no significant variations in the number of microbial cells. (C) Mutation accumulation: A reduction in the microbial population occurs, with each replicate, by streaking a culture onto a petri dish. Subsequently, a new colony is selected (originating from a single cell) and transferred to a new petri dish.

The main results regarding the application of ALE methods to different genera and species of LAB are shown in Table 1. These results will be discussed in the subsequent sections of this review.

3.1 Adaptive Laboratory Evolution methodologies

The modification of strains through natural strategies, such as ALE, is performed through the repeated selection of strains of desirable phenotypes, a method that, besides not requiring recombinant DNA technology, is generic and applicable to all microorganisms (Derkx et al., 2014). Different methods within ALE (Figure 2) can be employed to modify biotechnologically and industrially relevant microorganisms (Sandberg et al., 2019). In summary, ALE of microorganisms involves the selection of strains over long cultivation periods and under laboratory conditions. This step can be performed by simply transferring viable cells from one microbial culture to another flask containing fresh culture medium (Gresham & Dunham, 2014). Thus, ALE covers different techniques, among which the most common are serial dilutions (batch or fed-batch modes), chemostat, and mutation accumulation.

ALE experiments operating with batch and fed batch modes (Figure 2A) require the transfer of microbial cultures to the fresh culture medium at regular intervals. These experiments are easily reproducible since only common laboratory flasks (Erlenmeyer) are necessary. This method consists of cultivating bacterial cells for prolonged periods of time, under specific and desired conditions, in order to select strains that have acquired the ability to survive by developing spontaneous mutations (Gresham & Dunham, 2014; Sandberg et al., 2019). These experiments can also be carried out using a large number of replicates (McDonald, 2019).

Due to their ease of manipulation, it is possible to perform these experiments with high microbial populations (10^8 to 10^{10} cells). Cellular Division of these microorganisms can occur rapidly (20 min to 10 h/generation), for a few generations (100 to 500) (Sandberg et al., 2019). Thus, batch mode experiments are simple and the most widely used protocols for ALE and have been used in most of the articles cited in this review (Table 1) (Zhang et al., 2012; Chen et al., 2015; Ming et al., 2016; Kwon et al., 2018; Singhvi et al., 2018; Mladenović et al., 2019; Prasad et al., 2020; Liang et al., 2021; Liu et al., 2021).

The chemostat method (Figure 2B) allows tight control and maintenance of environmental conditions, extending the stationary phase of microbial growth (Gresham & Dunham, 2014). In this method, the culture medium is constantly added and the microbial culture, containing microbial cells, nutrients, and metabolites, is removed at the same rate (Lang et al., 2011, 2013; Gresham & Dunham, 2014).

Compared to batch and fed-batch modes, the chemostat method has some advantages. In the laboratory, chemostat cultures can be used to determine microbial growth rates and amounts of nutrients consumed and metabolites produced, as well as to allow the selection of strains with specific phenotypic functions (Hsu et al., 1977; Rajaraman et al., 2016; Zhang et al., 2021). Despite this, none of the articles described in this review used this method (Table 1).

ALE experiments can also be performed using the mutation accumulation method (Figure 2C), based on the serial propagation of microorganism colonies on Petri dishes, which generates a single-cell bottleneck at each replication of the microbial population, resulting in a significant decrease in cell concentration. In the case of mutation accumulation regarding ALE experiments, due to the loss of genetic variation, the new population can become genetically distinct from the original, which traces back to the theory of evolution (McDonald, 2019). Researchers can use this methodology in studies involving the accumulation of single mutations. However, to make the mutation accumulation method a form of laboratory adaptation, a step consisting of colony screening of colonies is necessary. Therefore, this methodology is less used and was not present in the articles described in this review. Taking into consideration that different ALE methods exist, the selection of which method to use depends on the objectives of the experiment. For example, the chemostat method is most indicated when, besides obtaining microbial strains with distinctive phenotypes and genotypes, greater degrees of control over the microbial population are sought.

3.2 Adaptive Laboratory Evolution to improve strains of *Lactobacillus*

Due to their ability to ferment raw ingredients, *Lactobacillus* is used in the production of yoghurt, cheese, beer, wine, and other fermented foods (Salvetti et al., 2012; Wang et al., 2017). Additionally, many *Lactobacillus* strains show probiotic characteristics (De Vuyst, 2000; Ming et al., 2016). During fermentation, these microorganisms are subjected to stress conditions due to factors such as temperature, pH, and osmotic pressure. For example, an increase in osmotic pressure across the bacterial cell wall occurs at the start of the fermentation process due to the high concentration of solutes in the medium, resulting in water efflux, dehydration, and consequent inactivation of enzymes relevant to the metabolism of these microorganisms (Mitchell et al., 2009; Liang et al., 2021).

Subsequently, during fermentation, variations in pH can occur due to the production of organic acids and changes in temperature. Thus, the selection of LAB must take into account their ability to resist or tolerate physical and chemical processes to which the microbial cells will be subjected during fermentation, aiming to maintain cell viability throughout the process (GuhanNath et al., 2014; Liang et al., 2021).

Due to the broad applications and versatility of *Lactobacillus* within the food industry, most of the articles selected for this review have applied ALE experiments for the adaptation of strains belonging to this genus. Authors have used ALE to enhance the following traits of *Lactobacillus*: increased lactic acid production and increased survival rate at acidic pH (Zhang et al., 2012; Chen et al., 2015; Mladenović et al., 2019; Liang et al., 2020), multiple stress tolerance (Ming et al., 2016), thermotolerance (Kwon et al., 2018) and antibiotic resistance (Wang et al., 2017). The *Lactobacillus* species most commonly used in the food industry are *Lacticaseibacillus rhamnosus, L. paracasei, L. casei, Lactobacillus delbrueckii* subsp. *bulgaricus, L. delbrueickii* (Li & Han, 2017). Therefore, different *Lactobacillus* species were subjected to ALE experiments (Table 1) to produce more lactic acid-tolerant strains.

Evolved strains of de *Lactiplantibacillus pentosus* CECT4023T after the adaptive process, produced higher amounts of lactic acid by consuming xylose (Cubas-Cano et al., 2019). Similarly, *L. paracasei* strains were subjected to ALE experiments in a molasses-rich medium and showed enhanced cell viability, in addition to producing more lactic acid than the original strain (Mladenović et al., 2019). Similar results were observed when using ALE to modify *L. casei* strains under acidic conditions (Zhang et al., 2012). The acid-tolerant strains showed higher cell viability than the original strain, in addition to higher lactic acid production under low pH values.

Table 1. Application of Adaptive Laboratory Evolution (ALE) on different strains of lactic acid bacteria: adaptive conditions, methodology, and main findings.

Lactic acid bacteria*	Adaptive conditions	ALE method	Parameters	Evaluations	Main findings	References
Lactiplantibacillus pentosus CECT4023T	20 g L^{-1} xylose 32 °C pH 7.0	Batch mode	Shaking: 150 rpm Cycles: NS Time between passages: 24 h Generations: 850	Lactic fermentation tests in bioreactor LA yield Xylose consumption	The evolved strain showed 2-fold more production of LA and 1.5-fold more consumption of xylose compared with the original strain.	Cubas-Cano et al., 2019
Lacticaseibacillus paracasei 4564	100 g L^{-1} sugar beet molasses pH 6.5 41 °C	Batch mode (first phase) Fed-batch mode (second phase) and pulsed fed-batch mode (third phase)	Shaking: 100 rpm Cycles: NS Time between passages: 20-24 h Generations: NS	Antioxidant activity DPPH Free Radical-Scavenging Activity LA-production capability	The evolved strain showed: one-fold higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging rates compared with the original strain. The LA production (89.4 g L^{-1}) of the evolved strain was superior to the original. The evolved strain showed a 59% higher capability to produce LA.	Mladenović et al., 2019

Table 1. Continued…

Lactic acid bacteria*	Adaptive conditions	ALE method	Parameters	Evaluations	Main findings	References
Lacticaseibacillus casei	37 °C pH 5.5, 5.0, 4.6, and 4.3 75 days	Batch mode	Shaking: NS Cycles: NS Time between passages: NS Generations: NS	Cell counts Intracelular pH Intracelular amino acid determination Lactate and acetate analyses Membrane permeability	The evolved strain showed a 318-fold higher survival rate than the original strain and exhibited a slightly higher production of lactate (13.6%) and 65.6% higher amount of acetate. The intracellular pH of the evolved strain was higher with lower inner membrane permeability. The survival rate of the evolved strain was increase in 1.36-, 2.10-, or 3.42-fold by the addition of 50 mM aspartate, arginine, and both, respectively.	Zhang et al., 2012
Lacticaseibacillus casei Zhang	Amoxicillin (0.5 µg/mL) and Gentamicin (1 µg/mL)	-	Shaking: NS Cycles: NS Time between passages: 24 h Generations: 1200	Evaluation of fitness as represented by minimum inhibitory concentrations (MICs) Identification of new mutations	The MIC for amoxicillin increased to a maximum level (8 µg/mL) for the evolved strains. There was a 4-fold increase in mutation accumulation (including SNVs, short insertions and deletions) in the evolved strains comparing to the control.	Wang et al., 2017
Lacticaseibacillus casei	37 °C pH 5.5, 5.0, 4.6 and 4.3 70 days	Batch mode	Shaking: NS Cycles: NS Time between passages: 12 h Generations: 50	Cell counts Bile tolerance Intracelular pH	ALE resulted in 1.6-fold higher biomass in evolved strain and higher resistance to bile salt. The evolved strain showed the capability of maintaining a higher pH compared to that of the original strain.	Ming et al., 2016
Lactobacillus delbrueickii Uc-3 (NCIM 5219)	pH 4.5	Fed-batch mode	Shaking: NS Cycles: 40 Time between passages: 48 h Generations: NS	LA concentration, productivity, and yield H+-ATPase activity determination Analysis of H+-ATPase gene expression by one-step quantitative real-time PCR	The evolved strain demonstrated a 1.80-fold increase in LA production, but its viability was reduced due to the acidic pH and/or end-product inhibition There were higher H+-ATPase activity and a 415-fold increase in H+-ATPase gene expression compared to the original strain.	Singhvi et al., 2018
Lacticaseibacillus rhamnosus	−30 °C 13 °C 37 °C	Batch mode	Shaking: NS Cycles: 150 Time between passages: 16 h Generations: NS	Viability Growth Dynamics Whole Genome Resequencing	The evolved strain viability increased up to 93.65%. The exponential growth rates were faster, with a 13-min shorter mean doubling time than the original strain. Six gene regions and an intergenic space, related with encoding exopolysaccharide biosynthesis protein that could be responsible for the strain adaptation were discovered.	Kwon et al., 2018

Table 1. Continued…

Lactic acid bacteria*	Adaptive conditions	ALE method	Parameters	Evaluations	Main findings	References
Lactobacillus delbrueckii subsp. bulgaricus ET45	37, 40 and 45 °C	Batch mode	Shaking: NS Cycles: NS Time between passages: NS Generations: NS	Enzymatic hydrolysis Production of D-LA by SHF	The enzymatic hydrolysis of the evolved strain showed 8% higher cellulose to D-LA conversion. There was an increase in the LA production by the evolved strain (108 g/L D-LA) and 60% conversion of cellulose to D-LA.	Prasad et al., 2020
Lactobacillus delbrueckii subsp. bulgaricus	52 °C	Batch mode	Shaking: NS Cycles: NS Time between passages: 48 h Generations: NS	Morphology Lactic acid production pH and viscosity Amino acids Volatile compounds	Changes were observed in the morphology of the evolved strain after ALE (changed from rod to filament (52 °C) to cocci after frozen storage). The evolved strain did not show LA production. The pH remains 6.84 ± 0.13. There was a 115- and 275-fold higher production of Arginine and Methionine (237.24 ± 5.94 and 98.83 ± 1.78 µg/100 g, respectively) by the evolved strain. The evolved strain showed an increase in diacetyl formation in milk.	Liang et al., 2021
Lactococcus lactis TM29	38, 39 and 40 °C	Batch mode	Shaking: NS Cycles: NS Time between passages: NS Generations: 860	Growth rate Physiological and transcriptomic characterization Next-Generation Sequencing	The evolved strain showed a 33% faster growth rate compared to the original strain and a 12% higher specific lactate production. Changes in the expression of single proteins (chaperone; riboflavin transporter) were observed. Two had pleiotropic effects (RNA polymerase) in the evolved strain after ALE. Deletion in 10 genes was also found to affect thermal tolerance significantly.	Chen et al., 2015
Lactococcus lactis	10 AU/mL of Lcn972 30 °C for 8 transfers	-	Shaking: NS Cycles: NS Time between passages: 16 h Generations: 850	Milk Acidification and Production of Lactic Acid Minimal Inhibitory Concentration Nisin Production Resistance to Antimicrobials	The evolved strain was capable to acidify the milk (pH below 5.3). ALE increased the MIC of Lcn972, between 4- and 32-fold. Nisin production was not compromised by ALE. Genes were involved in stress response, detoxification modules, cell envelope biogenesis and/or nucleotide metabolism.	López-González et al., 2018b

Table 1. Continued…

Lactic acid bacteria*	Adaptive conditions	ALE method	Parameters	Evaluations	Main findings	References
Lc. lactic ssp. *cremoris* MG1363	5 mM hydrogen peroxide 30 °C	Batch mode	Shaking: 200 rpm Cycles: NS Time between passages: 72 h Generations: NS	Determination of Bacterial Growth and Survival Acidification Test Vitamin K2 Extraction and Analysis Genome Sequencing Proteomics Analysis	The evolved strain showed a higher survival (dropped to 10^7 CFU/mL at 48 h from the initial 10^9 CFU/mL but further decreased to 10^4 CFU/mL at 72 h). ALE improved the production of vitamin K2 (50-110% increase) by the evolved strain. Common mutations in ldh and gapb genes were found in the evolved strain. Proteomics analysis showed that 16 proteins were differently expressed by the evolved strain: glyceraldehyde 3-phosphate dehydrogenase (A2RIN9), universal stress protein A2 (A2RK64) and form amidopyrimidine-DNA glycosylase.	Liu et al., 2021

*NS: not shown. LA: lactic acid; SNV: single nucleotide variation; D-LA: D-lactic acid; SHF: separate hydrolysis and fermentation;

Although previous studies found increases in both lactic acid production and cell viability, Singhvi et al. (2018) observed that *L. delbrueckii* Uc-3 strains (NCIM 5219), subjected to ALE, despite an increase in lactic acid production, showed a decrease in cell viability at pH 4.5. Thus, it is understood that each LAB species can develop its own adaptation mechanisms and, consequently, unique results will be found. These differences in results may be related to the increase in glycolysis and the supply of cofactors that are directly associated with lactic acid production, the pentose-phosphate pathway, and amino acid metabolism (Liang et al., 2020) in addition to the genetic fixation of mutants through altered gene expression (Sandberg et al., 2019).

In addition to industrial fermentative processes, acid stress is a common environmental condition encountered by LAB in the gastrointestinal tract. *L. casei* strains (Ming et al., 2016) were subjected to ALE for a period of 70 days, and three strains that showed enhanced tolerance to acidic conditions with a significant increase in biomass were isolated. The adapted strains also showed a higher survival rate in simulated gastric fluid (pH 2.5), intestinal fluid (pH 8.0), and bile salts. Tolerance to gastric and intestinal fluids and bile salts can be considered an important trait in *Lactobacillus* strains, allowing these microorganisms to survive and multiply with beneficial effects on the gastrointestinal tract. These results thus provide information on procedures that the food industry could adopt to select acid-tolerant strains to act as probiotics in foods. Another important characteristic of *Lactobacillus* strains is their tolerance to different temperatures, especially during the fermentation of dairy products. Among the various sources of stress, extreme temperature variations are an important factor in bacterial cell growth. However, few studies have used ALE for the development of thermotolerant LAB strains. Most studies still focus on microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (Caspeta et al., 2014; Sandberg et al., 2014), due to extensive knowledge about their genetic and molecular characteristics.

Among the articles cited in this Review, only three have used ALE experiments for the development of thermotolerant strains. Of these, one developed a thermotolerant strain of *L. delbrueckii* subsp. *bulgaricus* capable of converting cellulose into lactic acid, so that both saccharification and fermentation processes would occur simultaneously at 45 °C. The use of ALE resulted in an 8% increase in comparison to the

processes occurring in isolation (Prasad et al., 2020). This demonstrates that ALE can not only result in LAB adaptation towards a desired level of thermotolerance but also enhance the production of compounds of interest, such as lactic acid. After undergoing gradual temperature increments during the ALE experiments, *L. delbrueckii* subsp. *bulgaricus* strains were inoculated in milk in order to evaluate the production of aromatic compounds and lactic acid by the modified strains (Liang et al., 2021). However, the strains did not produce lactic acid and the pH of the milk remained between 6.0 and 7.0. Fermented milk usually shows pH values between 4.2 and 4.6. The decrease in pH triggers casein coagulation, resulting in a more consistent texture, which is an important sensory characteristic in yogurt, for example. Although they did not observe lactic acid production in milk inoculated with the modified *L. delbrueckii* subsp. *bulgaricus* strain, the fermented milk still had a firmer texture. According to the authors, this may have occurred due to the production of exopolysaccharides (EPSs) by LAB and their interactions with milk proteins.

However, they found that the evolved strain of *L. delbrueckii* subsp. *bulgaricus* showed high proteolytic activity (Liang et al., 2021). LAB proteolytic enzymes play an essential role in the conversion of milk proteins into aroma and flavor compounds, such as diacetyl, acetoin, and acetaldehyde. The aromatic compounds profile showed a significant increase in the production of diacetyl in milk after inoculation with the modified *L. delbrueckii* subsp. *bulgaricus* strain. Diacetyl is an industrially relevant aromatic compound, especially in the production of fresh or soft cheese (ripened for a short period of time). Its formation contributes to the sensory quality of the product, as it is easily identified by consumers due to its characteristic buttery aroma (Cheng, 2010). The increased production of this substance can be explained by the supplementation of the medium with lactose. This indicates that, when ALE experiments are performed with medium supplementation, a higher production of aromatic compounds by the LAB strains is induced, providing fermented products with specific aroma and flavor characteristics.

Low temperatures cause damage to microbial cells and hinder multiplication. However, *L. rhamnosus* strains that were subjected to 150 cycles of ALE experiments at -30 °C showed greater cell viability compared to the original strain (Kwon et al., 2018). Adaptation to low temperatures may be related to the loss of function of a few genes, which alter the microbial cell wall structure, resulting in increased membrane fluidity (Liechti et al., 2014; Kang et al., 2015; Kwon et al., 2018). The sequencing of *L. rhamnosus* strains modified through ALE resulted in variations to six genes involved in the biosynthesis of the cell membrane or wall (Liechti et al., 2014; Kang et al., 2015). This shows that the proper application of ALE promotes beneficial changes in tolerance-related genes so that the strain can adapt to new environmental conditions.

Gene expression is related to different stress response mechanisms in LAB strains (Zhang et al., 2012, 2015). Therefore, ALE is also applied in the investigation of molecular mechanisms related to bacterial survival under different stress conditions. The *L. casei* Zhang were subjected to antibiotic stress with amoxicillin and gentamicin, and genetic variations were investigated (Wang et al., 2017). After adaptation, the *Lactobacillus* strains showed an increase in minimum inhibitory concentration values against amoxicillin and different mutations due to the presence of antibiotics. One of the main mechanisms that allows a microorganism to become resistant to an antibiotic is the selection of mutant subpopulations that carry genes capable of effectively inactivating the antibiotic (Woodford & Ellington, 2007). However, it is not possible to know whether this is a species-specific or a universal feature in all LAB (Alekshun & Levy, 2007; Wang et al., 2017).

The results presented here are evidence that ALE can contribute to improving *Lactobacillus* strains of industrial interest. In addition to the enhancement of survivability traits of a microorganism, ALE can develop *Lactobacillus* strains that result in fermented products with unique flavor, aroma, and texture properties. Therefore, the selection of LAB strains that are tolerant to multiple types of stress and in possession of specific traits can help increase productivity and, consequently, reduce costs associated with fermentative processes in the food industry.

3.3 Adaptive Laboratory Evolution to improve strains of *Lactococcus*

Lactococcus are subjected to multiple types of stress, such as heat, oxidation, acidic pH, and salinity during processes within the fermented foods industry (Chen et al., 2015). For example, temperatures can reach 40 °C during milk protein coagulation in cheese production, which hinders bacterial growth and, consequently, lactic acid production (Chen et al., 2015; Treguier et al., 2019).

Chen et al. (2015) subjected a *Lactococcus lactis* strain to ALE (Table 1) in order to improve its survivability at temperatures close to 40 °C. The authors found a significant increase in the survival rate of the evolved strain at different temperatures (38, 39, and 40 °C). The whole genome sequencing of the modified strains revealed the presence of genes associated with heat tolerance. Similar to what was reported in studies on *Lactobacillus*, Chen et al. (2015) also observed that ALE-modified *L. lactis* strains produce more lactic acid and, therefore, acidify faster than the original strain when grown in a chemically defined medium.

López-González et al. (2018a) exposed both industrially sourced and wild nisin-producing *L. lactis* strains to different concentrations of the bacteriocin Lcn972. The authors observed a 4- to 32-fold increase in the inhibitory concentration of this biocomposite compared to the respective original strains. Furthermore, modified *L. lactis* strains were able to acidify the milk to pH values below 5.3. They also found mutations in genes involved in stress response, detoxification, cell envelope biosynthesis, and nucleotide metabolism. These results demonstrate that both the phenotypic and genetic characterization of adapted microorganisms generate relevant data regarding the applications of these strains in the fermented food industry.

ALE can also be used to enhance the ability of LAB to produce micronutrients such as vitamins. Vitamin K2 acts as a cofactor for enzymes involved in biological processes such as blood coagulation, calcium metabolism, and cell growth (Vermeer & Schurgers, 2000; Vermeer, 2012; Liu et al., 2021). The human diet can be enriched with vitamin K2 through the incorporation of bacteria such as *L. lactis* into fermented foods. In the study conducted by Liu et al. (2021) strains of *L. lactis* ssp. *cremoris* MG1363 were cultured under oxidative stress. After 100 generations, sequencing of selected strains revealed common mutations in the genes ldh and gapB. Proteomic analysis showed an overproduction of glyceraldehyde 3-phosphate dehydrogenase (GapA), universal stress protein A2 (UspA2), and formamidopyrimidine-DNA glycosylase (MutM), proteins with putative functions in redox reactions, universal stress response, and DNA damage repair, which can contribute to the improved resistance towards oxidative stress.

Similarly to *Lactobacillus*, *Lactococcus* is widely used microorganisms in the fermented foods industry, especially in the manufacturing of dairy products. Therefore, the development of *Lactococcus* strains with enhanced tolerance to different temperature and pH conditions is indispensable to the production of fermented goods with unique characteristics.

3.4 Present and future of Adaptive Laboratory Evolution

In the studies cited in this Review (Table 1), modifications were made to the LAB strains in terms of lactic acid production capacity, accompanied by improved tolerance to acidic environments (Zhang et al., 2012; Chen et al., 2015; Mladenović et al., 2019; Liang et al., 2021), tolerance to high and low temperatures (Kwon et al., 2018), antibiotic resistance (Wang et al., 2017), the production of aromatic compounds (Liang et al., 2021) and vitamins (Liu et al., 2021). In some studies, alterations to metabolism and specific regions of the DNA of LAB were also reported (Kwon et al., 2018; Liu et al., 2021), besides the developing or enhancing multiple-stress tolerance (Ming et al., 2016). However, only one of the studies reported the application of the evolved LAB strain in a food product (milk) in order to determine the effects provided by the evolved strain on the characteristics of fermented milk (Liang et al., 2021). In this sense, there is a technical-scientific gap in terms of the application of ALE-modified LAB strains in the preparation of fermented foods.

Nevertheless, ALE is a promising methodological alternative for the improvement of LAB strains of industrial interest. Furthermore, it is expected that the interest in the aggregation of genetic and phenotypic analysis, as well as the interest in *in silico* evolutionary experiments, will grow. These evaluations should allow for more detailed analyses of complex phenomena and, in turn, stimulate the implementation of evolutionary experiments on increasingly sophisticated microorganisms. ALE offers technological and scientific advantages in the development and enhancement of strains in addition to process optimization. Therefore, through these methods, LAB can be used more efficiently within the food industry thanks to greater control over the process and, consequently, the overall quality of the final product.

ALE experiments can be performed simply via serial dilution of bacterial cultures or continuous culture (chemostat) methods. However, some limitations such as the time-consuming and labor-intensive manual transfer of cultures in batch mode cultivation as well as the difficulty of precisely controlling the cultivation parameters should be considered (Wu et al., 2022). The characterization of mutations developed during the adaptation process also requires significant effort (Derkx et al., 2014), but recent advances in the automation of ALE experiments should eventually overcome these technical obstacles (Johansen, 2018; Horinouchi & Furusawa, 2020).

To provide a better understanding of the mutations occurring during ALE experiments, a free-access web platform called ALEdb (aledb.org) has been launched. The database offers information about ALE experiments, evaluated conditions, and published studies. ALEdb provides tools that allow users to search for specific mutations, report key mutations, and export mutation data for custom analysis. With these tools, ALEdb aims to fill gaps in the field of experimental evolution by being an accessible and valuable resource of established adaptive experiments (Phaneuf et al., 2018; Bojar, 2019).

The expansion of ALEdb could generate a useful platform for the exploration and usage of the principles of evolution, with the inclusion of non-native genes and mutagenesis conditions, incorporating the full range of data obtained from evolutionary experiments. There are also methodologies and equipment for automating ALE experiments. A morbidostat can detect population increases and adjust substrate levels acting as selection pressure (Lee & Kim, 2020). A fluorostat, on the other hand, combines fluorescence detection with a turbidostat and can be applied to determine levels of gene expression (Toprak et al., 2012; Takahashi et al., 2015; Lee & Kim, 2020).

Furthermore, eVOLVER, a framework composed of software and hardware that can customize experimental conditions according to a specific purpose and that can be used as a tool that can be reconfigured in the laboratory, was also released in recent years (Wong et al., 2018; Lee & Kim, 2020). In this way, these techniques can be applied for faster and more accurate experiments, and the results obtained from these can be applied industrially.

Several automated culture platforms developed with the automation of ALE experiments in mind are also on the market (Wong et al., 2018; Wu et al., 2022). For example, iBioFAB was developed to achieve automation of all stages related to microbial engineering. It can house over 500 96-well plates and also automatically perform ALE experiments with *in vitro* mutagenesis methods (Si et al., 2017; Wu et al., 2022). However, it is not affordable for small research laboratories.

Other options are platforms capable of performing ALE experiments using *in vivo* mutagenesis methods, such as the milliliter-scale Mini Pilot Plant and the microliter-scale microbial microdroplet culture (MCC) system (Jian et al., 2020; Wu et al., 2022). The Mini Pilot Plant can monitor pH, dissolved oxygen, and biomass of cultures in FlowerPlates® (Wu et al., 2022). Thus, we can assume that, in the last decade, significant advances have been made to improve ALE techniques in order to decrease the time consumed by these methods while also ensuring the ability to fully monitor the experiments.

A growing number of studies demonstrated the selection of new LAB strains with different functional traits, such as tolerance to stress and the production of different biomolecules, such as enzymes, polysaccharides, and aromatic compounds. However, some gaps still exist and should be filled by studies aimed at improving LAB strains through ALE experiments that do not involve the genetic manipulation of these microorganisms, due to the higher acceptability of the incorporation of this type of bacteria in food products by consumers.

4 Conclusions

Adaptive Laboratory Evolution (ALE) has shown to be a promising approach for food biotechnological processes because it enables the enhancement of lactic acid bacteria (LAB) strains and is effective in developing or obtaining desired phenotypes without the need for direct genetic modifications. However, continued efforts are needed to understand all the mechanisms employed by LAB during ALE experiments. In addition, technological advances aimed at process optimization and higher levels of control over all ALE steps are essential to continuously improve the technique.

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