Screening of Lactic Acid Bacteria strains for biopharmaceutical-grade plasmid and recombinant protein production

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Abstract | Lactic acid bacteria (LAB) have gained increasing interest in the fields of biotechnology and biopharmaceuticals as they offer needed characteristics to the industry for the creation of DNA vaccines and recombinant protein production. When compared to other enteric bacterial strains such as *Escherichia coli*, LAB shows promise as they are regarded as safe due to its non-pathogenic nature. Challenges on their efficiency as microbial cell factories were found, as *Lactococcus lactis* subsp. *lactis* LMG 19460 showed disappointing recombinant protein and plasmid DNA yields in previous studies. This created the need to consider several promising strains of *Lactococcus* (3) and *Lactobacillus* (4) as better delivery systems for these applications. The screening of temperatures (30ºC, 37ºC, 40ºC, 43ºC), media (M17 ad MRS) and agitation (0, 100 rpm) conditions allowed the establishment of the optimal growth conditions for each strain, considering the growth rate and maximum OD_{600} obtained. Antibiotic susceptibility screening was performed and demonstrated a high resistance (>2000 µg/mL) of all strains to Kanamycin, Neomycin, Apramycin and Spectinomycin except the two *L. cremoris* strains tested in which the resistance was significantly lower (200-500 µg/mL). Erythromycin and Chloramphenicol showed particularly low minimal inhibitory concentration (MIC) values making them appropriate to use as resistance markers. Transformation feasibility was analyzed by electroporation, to evaluate which strain delivered the highest yield and best quality pDNA. Transformation in one strain – *Lactococcus lactis* ssp. *cremoris* MG 1363 – was capable to produce higher pDNA concentrations when compared to our reference strain (LMG 19460). Evidence from this work suggests that a more suitable candidate is available, and further screen of its physiological and molecular properties can lead to an efficient gene expression protocol with a high range of applications in the fields of bioengineering

Introduction

Lactic acid bacteria (LAB) play an important role in food, agricultural, and clinical applications. LAB form a phylogenic heterogeneous group of Gram-positive bacteria unified by their morphological, metabolic and physiological characteristics[1], mainly defined by their production of lactic acid as the main or sole product of carbohydrate fermentation.[2] Described as facultatively anaerobic and aerotolerant, they test negative for oxidase, catalase and benzidine, lack cytochromes and are acid tolerant. LAB usually consist in nonmotile asporogenous rods and cocci bacteria that are unable to utilize lactate.[3]

Given that the definition of this group is biological rather than taxonomical, LAB do not represent a monophyletic group of bacteria and are widely distributed in nature[4]. Morphology, mode of glucose fermentation, reaction to different growth temperatures, agglutinations, ability to grow at high salt concentrations, acid or alkaline tolerance and the form of the lactic acid produced (D, L or both), have been a few of the characters that remain essential to the current LAB classification system[5]. With the availability of improved molecular methodologies such as 16S rRNA gene sequencing and studies on the microbial diversity of different environments, many species have been discovered and identified[2]. Thus, the heterogeneous LAB group consists in the main genus *Lactobacillus, Lactococcus, Pediococcus, Streptococcus* and *Leuconostoc*[4].

Being generally recognized as safe (GRAS) – by their non-invasive and non-pathogenic nature; ability to endure the gastrointestinal tract and to colonize mucosal tissue [6]; and the fact that they're indigenous to food-related environments and can also be associated to mucosal surfaces of animals – this group of bacteria is one of the oldest forms of preservation, widely used in the food industry,

also giving unique features like flavor, texture and nutritional value of these products and novel applications as bio-protective, probiotic cultures have also been of relevance, as they are proven to have a beneficial effect on the health and well-being of the host [7].

Production of recombinant proteins in microbial systems and DNA based vaccines have revolutionized biochemistry and the biopharmaceutical industry since the beginning of the century. Given the need to develop an alternative route for the administration, as well as a safety delivery platform, LAB have been extensively investigated as a delivery vector for therapeutic proteins, DNA and vaccine antigens, given they are generally regarded as safe (GRAS) and association with probiotic characteristics, their capacity to survive the passage through the gastrointestinal tract, together with the gradual availability of tools for genetic modification led to its increasing use for novel medical applications [8].

These challenges presented their self as the most used plasmids for this application are produced by E. coli cells which produce lipopolysaccharides that can co-purify with pDNA and generate inflammatory responses[9]. Thus, studies with LAB have gained increasing attention.

From the diversity of LAB species, *Lactococcus lactis* and some species of the *Lactobacillus* genus, have been the most widely used considering cloning and production of recombinant proteins and DNA [1,4]. *L. lactis* has been deeply characterized, being the first one whose genome was fully sequences and it consists on an expression system easy to manipulate with several cloning and expression systems available. For the expression of heterologous genes in LAB, several plasmids have been developed, but their copy numbers are generally very low. Thereafter, it is not easy to achieve high-level gene expression using those low-copy plasmids, and the limited availability of plasmids has been one of the big hurdles in using LAB as protein production hosts. To solve this problem, these bacteria may benefit genome editing techniques to achieve higher plasmid yields either for gene therapy purposes or increased protein, as they produce much less amount of plasmid then other enteric bacteria such as E. coli and present a high level of pDNA degradation at a fast rate [9].

The current protocols utilized in order to obtain a better delivery system for lactic acid bacteria have been focusing on the *Lactococcus lactis* species [10].

Materials and Methods

Bacterial strains and growth conditions **|**
The bacterial strains used in this study are listed in **Table 1**.

Strain	Characteristics	Source
Lactococcus lactis* subsp. *lactis LMG 19460	Plasmid Free*	BCCM Culture Collection, Belgium
Lactococcus lactis* subsp. *lactis IL1403	Plasmid Free*	Institut Micalis, France
Lactococcus lactis* subsp. *cremoris KW2	Wild Type, Plasmid Free	Louvain Institute of Biomolecular Science and Technology, Belgium
***Lactococcus lactis* subsp. *cremoris* MG1363**	Plasmid Free*	University of Groningen, Netherlands
Lactobacillus plantarum CCUG 61730	Wild Type, Plasmid Free	Collection of Culture University of Göteborg, Sweden
Lactobacillus casei ATCC 393	Wild Type, Plasmid Free	American Type Culture Collection, EUA
***Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842**	Plasmid Free*	American Type Culture Collection, EUA
***Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA 365**	Wild Type, Plasmid Free	American Type Culture Collection, EUA
***Escherichia coli* DH5α**	recA- and endA-. Produces methylated DNA	Invitrogen
***Plasmid* pTRKH3**	Original E. coli/LAB shuttle. EryR. 7766 bp	BCCM/LMBP Plasmid Collection

Table 1 - Bacterial strains and plasmid used for the screening and electroporation protocol, as well as their characteristics, origin and source. (* Strains are plasmid-free by distinct curing strategies applied to their parental strains).

For *Lactococcus lactis* strains the growth conditions considered were 30ºC with a 100 rpm agitation, in M17 broth medium from SIGMA-ALDRICH supplemented with 20g/L glucose (M17-G) in 15 mL falcon tubes with 5 mL medium, and *Lactobacillus* strains were grown in MRS medium, at 37ºC without agitation.

The M17 medium is composed by ascorbic acid 0.5 g/L, lactose 5 g/L, magnesium sulfate 0.25 g/L, meat extract 5 g/L, meat peptone 2.5 g/L, sodium glycerophosphate 19 g/L, soy peptone 5 g/L, tryptone 2.5 g/L, and yeast extract 2.5 g/L and the MRS medium from Liofilchem is composed by peptospecial 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, glucose 20 g/L, Triammonium Citrate 2 g/L, Sodium acetate 5 g/L, magnesium sulfate 0,2 g/L, manganese sulfate 0,05 g/L and dipotassium phosphate 2g/L. It is necessary to add 1 mL/L of Tween 80 to the MRS medium preparation.

E. coli DH5α used for plasmid extraction were grown at 37ºC, 250 rpm, in LB medium (Nzytech) supplemented with 500 µg/mL erythromycin (Sigma) for maintenance of the plasmid during growth.

Cell frozen stocks **|** Bacterial cell stocks were prepared using 80 µL cell suspension solution and 20 µL glycerol 99.99% and preserved in -80ºC for storage. As glycerol is toxic to most cells, weakening their cell walls, cell preparations must be saved on ice immediately until -80ºC preservation.

Growth conditions optimization **|** The strains were grown in a pre-inoculum overnight, in their standard conditions according to the strain, in 15ml falcon tubes contain 5ml of the correspondent medium. These overnight pre-inoculum were used to inoculate with a starting Optical density at 600 nm (OD_{600}) of 0.1, in new 15ml falcon tubes contain 5ml of new fresh medium.

The analysis of growth of the five LAB strains was performed in a range of four temperatures – 30, 37, 40, 43 (ºC) in both media – M17 + 20g/L glucose and MRS. All tubes were incubated at 100 rpm. Monitoring the growth was made in a scattered manner, allowing the follow up until 30h of growth, necessary mainly in MRS cultured tubes. Optical density (OD_{600}) was measured in regular timespans of 2-3 hours until the stationary phase was achieved, and the OD_{600} values were plotted in the Logarithmic scale (Log_{10}) versus time(h), as bacterial cultures grow exponentially. pH levels were measured at the end of growth (24-30h) and the values compared to the initial pH value measured for both fresh media utilized. For the optimal growth conditions of temperature and media stablished for each strain, the same protocol was implemented to compare the growth without agitation.

Optimized growth conditions | For further work in this paper, the considered optimal conditions are:

• Growth in MRS medium, at 30ºC, 100 rpm for *L. lactis* subsp. *lactis* strains

• Growth in M17-G medium, at 30ºC, 100 rpm for *L. lactis* subsp. *cremoris* strains

• Growth in MRS medium, at 37ºC, 0 rpm (although not compulsory), for the four *Lactobacillus* strains.

Antibiotic susceptibility profiling **|** The antibiotics tested in this study were Erythromycin, Ampicillin, Kanamycin, Neomycin, Chloramphenicol, Apramycin and Spectinomycin. Two 24-well microplate platforms were used to test the grow the LAB strains in a gradient of antibiotic concentrations listed in **Table 2.**

Antibiotic	Concentrations tested (µg/mL)
Erythromycin	5, 25*, 50, 100
Ampicillin	25, 50, 75, 1000
Kanamycin	250, 500, 1000, 2000
Neomycin	250, 500, 1000, 2000
Apramycin	250, 500, 1000, 2000
Chloramphenicol	(2.35; 4.7)**, 9.4, 18.8, 37.5, 75***, 600***
Spectinomycin	25, 50, 100, 200

Table 2 - Concentrations to test antibiotic susceptibility of LAB strains * The well as used for the negative control. Concentration not tested; ** Concentrations tested after the higher concentrations failed to show a MIC value. *** Concentrations excluded for failing to show the MIC value.

For this purpose, cell banks of the strains were grown, in 15ml falcon tubes containing 5ml of correspondent media, overnight at the optimized growth conditions for each one. The different pre-inoculums were then used to start a new culture by the inoculation to the wells with 3.75ml of correspondent media up to OD_{600} =0.1. After, the microplates were incubated, one at 37ºC with no

agitation – for the *Lactobacillus* strains; and 30ºC with agitation (100 rpm) for the *Lactococcus* strains. The optical density of the growth was measured at 24 and 48 hours of growth.

***Cell transformation protocol* |** This method was based on the protocol prepared by Holo & Nes, (1989), modified to enhance transformation efficiency of *Lactococcus lactis* LMG19460. Cells were made electrocompetent by the addition of glycine, that acts as a cell weakening agent. A stationary phase culture of the recipient strain was inoculated, in a set volume of culture, into 75 mL M17-G broth. The cells were then incubated (30ºC, 100 rpm) until mid-log phase of growth (OD_{600} 0.5-0.8) and transferred (750µL) to a new flask of M17-G medium supplemented with sucrose 5M and either 1% or 2% glycine (v/v). Flask grew to an OD_{600} of at least 1.0, cells and harvested by centrifugation (6000 xg, 6', 4ºC) and washed twice with 1 mL washing solution (WS) (glycerol 10% (v/v); sucrose 0,5M). Final pellets were resuspended in 1/100 culture volume of WS and then distributed to 80 µL aliquots. For electroporation the stored aliquots were diluted in the appropriate volume PCR grade water (filtered and autoclaved) for all electrocompetent cells strains to contain approximately the same number of cells ($1x10^{9}$*). Portions (40 µL) were mixed with plasmid DNA (pTRKH3) and transferred to a pre-cooled 1mm gap 90 µL electroporation cuvette. After 30 min on ice, three electrical pulses were delivered by electroporator of 1kV, 25 µF and 400Ω, resulting in time constants of 8 to 9 ms. Rapidly after, the suspension was diluted with 960 µL Recuperation Medium (M17-G, sucrose 0.5M; MgCl2 20 mM, CaCl2 2 mM) and left on ice for 5 minutes. Followed by a 3 hours, at 30ºC for *Lactococcus* strains and at 37ºC for *Lactobacillus*, to allow recovery, cells were then collected by centrifugation, resuspended and used to inoculate 5 mL M17-G supplemented with a sub-lethal erythromycin concentration (2.5 µg/mL), (ON, 30ºC, 100 rpm). After growth, OD_{600} was measured for each tube and the cells centrifuged (6000 xg, 3', 4ºC) and plated on Regeneration medium (RgM) supplemented with 5 µg/mL erythromycin. Transformants were enumerated after 2 days of incubation. Regeneration medium composition is Tryptone 10 g/L, Yeast Extract 5 g/L, Sucrose 200 g/L, glucose 10 g/L, Gelatine 25 g/L, Agar 15 g/L, MgCl2 2.5 mM, CaCl2 2.5 mM. The transformation efficiencies were expressed as transformants per microgram of plasmid DNA. Control experiments were performed by plating cells which were electroporated without plasmid DNA.

For the electroporation of LAB strains following the optimal conditions, changes in the protocol were made in order to adjust the steps to the medium, temperature and agitation settings more adequate for each specific strain. Dilution volume was adjusted to match the same cell number of transformants per aliquot of electrocompetent cells to the value of reference (for LMG19460).

***Plasmid purification, quantifications and quality assessment* |** Transformant colonies were peaked and inoculated in 5 mL of medium (M17-G or MRS) supplemented with 5 µg/mL erythromycin, overnight at 30ºC or 37ºC, 100 or 0 rpm for *Lactococcus* or *Lactobacillus* respectively. Cell frozen stocks were made for storage, and the remaining volume centrifugated (6000 xg, 3', 4ºC). Nucleo Spin® Plasmid, DNA, RNA and protein purification kit, from Macherey Nagel Bioanalysis was used to extract plasmid DNA. The first two steps were based on "Isolation of plasmids from Gram-positive bacteria" and for the remaining purification, the protocol "Isolation of low-copy plasmids" was considered.

Plasmid DNA was quantified by Nanodrop measurement (Nanovue Plus, GE) and its quality assessed by agarose gel electrophoresis. 1 µL was used for nanodrop quantification, using PCR grade water for calibration. To confirm the presence and quality of the desired extracted pDNA, 500 ng to 1 µg of DNA was run in agarose gel for visualization.

Results and Discussion

Culture growth conditions optimization of Lactococcus and Lactobacillus strains |

Lactic Acid Bacteria strains are reported to be very fastidious organisms with several growth requirements generally present in complex media like MRS and M-17, which contain several compounds like amino acids, peptides, vitamins and nucleic acids[11]. Since MRS and M-17 media have been extensively used in our laboratory for the growth of *L. lactis* LMG19460, these two media were selected to perform the cell growth optimization. The temperatures analyzed – 30ºC, 37ºC, 40ºC and 43ºC – are in between the range of temperatures considered as ideal in laboratory

work regarding the use of several LAB, and for that considered suitable to be tested in our LAB strains.

This screening for the optimal medium, temperature and agitation conditions was made under the hypothesis that a higher cell division rate can be directly linked to a higher plasmid production when it comes to transformation.

In this screening, the parameters of interest after obtaining the growth curves were: the maximum OD600 obtained, the pH variation, the growth rate (μ) calculated from the exponential phase of the curve and the duplication time for each growth. For all the conditions tested, none of the values seem to have an inhibitory or limiting effect on the growth of either strain.

Culture media |

The optimized growth conditions obtained showed a preference of most strains to the MRS medium, except for *L. cremoris* strains that grew better with M17-G. (Fig 1(a)). In general, the exponential phase is reached sooner when in M17-G, but higher OD_{600} values are obtained in MRS.

Temperature |

Response seems to be interconnected to the medium used as it was observed that sensibility to temperature varies depending on this factor. Specific growth rates of each strains at each temperature is observed in the **Figure 1 (a)** and **(b)** for the two media analyzed.

Lactococcus strains (**Figure 1 (a))** prefer tepid temperatures - 30ºC for *L. lactis* subsp. *lactis* IL 1403 and *for L. lactis* subsp. *cremoris* MG 1363, and 37ºC for *L. lactis* subsp. *lactis* LMG 19460 and *L. lactis* subsp. *cremoris* KW 2, although this preference comes with different media preferences. The strain with the highest specific growth rate was MG 1363 that obtained a 1.02 h^{-1} rate in M17-G, 30ºC.

Lactobacillus strains (**Figure 1 (b))** seemed to prefer 37ºC. With Variation of the growth profile is similar between temperatures, and again seems to be medium-dependent. Comparatively to *Lactococcus* strains, this group of strains are heat resistant, specially the Lb. *delbruackii* strains, that belong to the termobacillus group of the classical LAB classification. However, *Lactobacillus casei* subsp. *casei* ATCC 393 seems to be less resistant when compared to *L. lactis* strains LMG 19460 and IL 1403. This may be explained by the fact that this *L. casei* is the only strain of our *Lactobacillus* strains that is commonly found in the gut and not in the food industry.

For the remaining *Lactobacillus* strains, the difference in the specific growth rate (μ) in each temperature doubles in average, between the two media used.

The growth analysis at 43ºC was made in more detail (data not shown) as the strain might need to endure high temperatures for application of genome editing techniques. Noteworthy for the difference are *L. cremoris* strains that achieve a significantly higher specific growth rate in M17-G

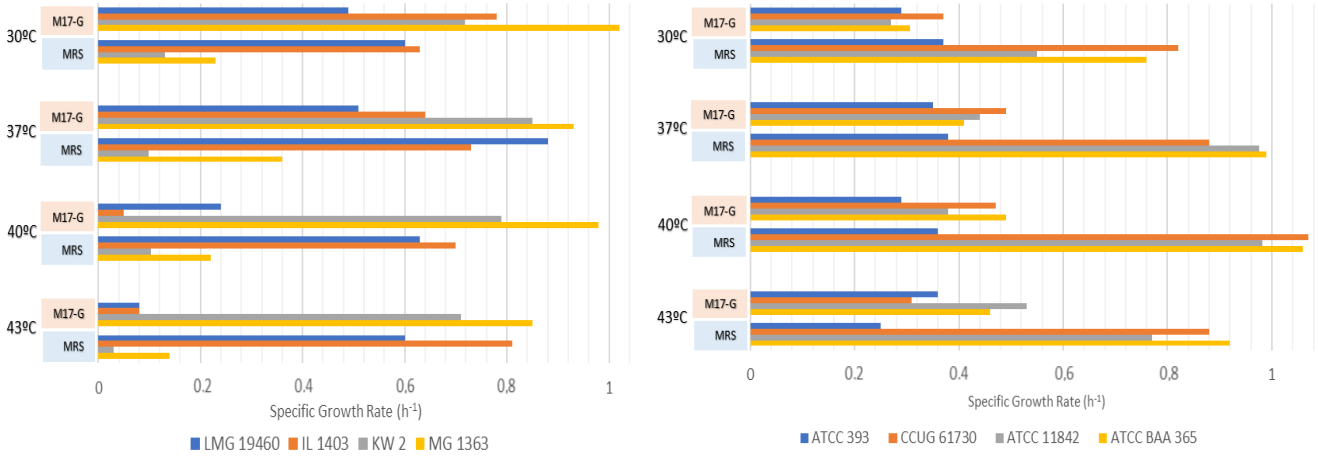

Figure 1- Comparative analysis of temperature effect (30º, 37º, 40º, 43ºC) on specific growth rates (h-1) of **(a)**- *Lactococcus* strains on the left and **(b)** *Lactobacillus* (right), in both culture media tested (M17-G and MRS) in 100 rpm

and the maximum OD600 reached for MG 1363 is higher in M17.

Agitation |
The growth curves showed no clear preference in the presence or absence of a mild agitation (100 rpm) of the falcon tubes. However a slight preference of *Lactococcus* towards a mild agitation and of *Lactobacillus* to no agitation and perceived and taken as optimal for the correspondent strains.

Optimal Growth conditions | *Lactococcus* and *Lactobacillus* strains are separated by their preferences in temperature – 30ºC and 37ºC, respectively. However, a division within the *Lactococcus* genus exists as the species *L. lactis* and *L. cremoris* differ in their medium of preference, as strains KW 2 and MG 1363 showed a more stable and faster growth in M17-G, and in *L. lactis* strains MRS was the best medium concerning final cell yield. Regarding agitation, *Lactococcus* shows an inclination towards a mild agitation preference in contrast to *Lactobacillus*, particularly the yogurt derived strains *L. bulgaricus*. However, from the three parameters analysed, agitation absence or presence had the least impact on growth rate and cell density obtained.
This growth conditions were used in the remaining work, with the *Lactococcus* strains growing in either MRS (*L. lactis* strains) or M17-G (*L. cremoris* strains) at 30ºC with a mild agitation of 100 rpm, and *Lactobacillus* strains with MRS medium, 37ºC and no agitation.

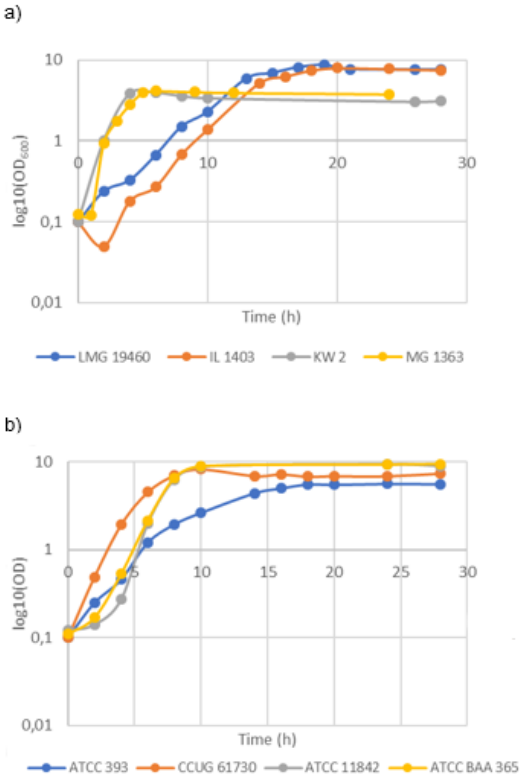

Figure 2- Optimized growth conditions established for each LAB strain (a) *Lactococcus* and (b) *Lactobacillus*, screened in this work. Growth rate (h-1), duplication time (h), Maximum OD600 obtained at the end of the exponential phase and at the end of growth were considered to establish these parameters.

Antibiotic susceptibility profile

The minimum inhibitory concentration (MIC) range of resistance for the LAB strains is shown in **Table 3** (a) and (b). For each strain, the presence of growth in a given well represents the antibiotic resistance of the strain at that concentration and the lack of growth (near an OD600 of 0.1) indicates the MIC value of the strain for the antibiotic.
All strains showed susceptibility to erythromycin, ampicillin, and chloramphenicol. Although the minimum inhibitory concentration was not defined in an exact value. These antibiotics can then be used as resistance markers in transformation selection and other bioengineering techniques.

Strains	Ery	Amp	Cn
LMG 19460	< 5	< 25	2.35
IL 1403	< 5	< 25	< 2.35
KW 2	< 5	< 25	< 2.35
MG 1363	< 5	< 25	< 2.35
ATCC 393	< 5	< 25	9.4
CCUG 61730	< 5	< 25	< 2.35
ATCC 11842	< 5	< 25	< 2.35
ATCC BAA 365	< 5	< 25	< 2.35

Table 3(a) - Antibiotic susceptibility profiling of *Lactococcus* and *Lactobacillus* strains. The MIC values (µg/mL) obtained are approximate and represents the concentration of antibiotic needed for no growth – measured by OD_{600} –to be observed

High resistance was found for kanamycin, neomycin, apramycin and spectinomycin, all belonging to the aminoglycoside class or similar (spectinomycin). Most strains revealed a resistance greater than or near 2000 µg/mL.
Strains of the species *L. cremoris,* however, have a MIC for kanamycin lower that the minimum concentration tested (250 µg/mL) and show similar response to apramycin, an antibiotic of the same class – aminoglycosides. The range of inhibitory effect were also possible to observe in these strains for spectinomycin, with a MIC between 50-200 µg/mL.

Strains	Kan	Neo	Apra	Spec
LMG 19460	> 2000	>2000	>2000	>200
IL 1403	2000	>2000	2000	>200
KW 2	< 250	>2000*	250	50-200
MG 1363	< 250	>2000	< 250	50-100
ATCC 393	>2000	>2000	2000	>200
CCUG 61730	>2000	>2000	>2000	>200
ATCC 11842	1000 – 2000	>2000	2000*	>200
ATCC BAA 365	1000*	>2000	>2000	200

Table 3(b) - Antibiotic resistance profiling of *Lactococcus* and *Lactobacillus* strains. The MIC values (µg/mL) obtained are approximate and represents the concentration of antibiotic needed for minimal(*) or no growth – measured by OD_{600} –to be observed

The antibiotics that showed susceptibility can then be used as resistance selection markers in transformation and other bioengineering techniques. However, for the use of LAB in the application of DNA vaccines as well as other applications involving introduction of live bacteria into the human organism, the use of antibiotic markers is generally not ideal and even prohibited since which jeopardizes its GRAS status [12].However, genome editing with antibiotic selection markers can be applied for strain advancement in an indirect way as a research tool, by improving knowledge on the strain itself and the relations with its hosts, as well as provide guidance towards targets for modifications using 'natural' or accepted editing methods avoiding a GMO label[13].

Transformation of the selected strains with plasmid pTRKH3

Transformations were firstly performed as described by Duarte (2018), applying the standard growth conditions for LMG19460 in all strains – 30º, M17-G medium and 100 rpm. Afterward, as the optimization studies showed that some strains had better growth in different temperature and medium settings, these changes were applied to the transformation protocol. The results obtained from the transformation for LMG 19460, our reference strain, are considered for comparison to the transformation on other strains, and in other conditions, as our main purpose is to uncover a strain that responds better to transformation and produces plasmid in greater quantity and stability.

***Lactococcus* strains |** Transformation in the standard conditions (30ºC, M17-G, 100 rpm) was successful, as expected, in *L. lactis* LMG 19460, either in cells treated with 1% and 2% glycine. Colonies also appeared on plates for *L. lactis* IL1403 (1% glycine) and for *L. cremoris* MG1363 (in 1% and 2% of glycine). Transformation efficiencies (TE) were calculated, representing the number of colonies (CFUs) produced by transforming 1µg of plasmid into a given volume of competent cells.
For LMG19460 the best TE obtained was of 6.8x 10^7 CFU/ µg pDNA for 1% glycine and 1.2x10^8 CFU/ µg pDNA for 2%. Comparing to these values, IL1403 obtained 1.6x10^6 CFU/ µg, and only in one of the three (3) plates incubated. *L. cremoris* MG1363 was able to transform the plasmid pTKRH3 at a similar efficiency on the order of 10^7 for 1% glycine treated cells and 10^8 in the 2% glycine.
After colony count and efficiency assessed, transformant colonies were peaked for growth in liquid medium (M17-G supplemented with erythromycin 5 µg/mL) to replicate the introduced plasmid to onwards proceed to its purification.
L. lactis IL 1403 has capable to grow colonies in plate but unable to grow in liquid medium after transformation. As incompatibility with the pTRKH3 plasmid would not result in transformant colonies after plating, we assume that the colonies obtained are, most likely, false positives obtained for IL 1403 that the concentration of antibiotic was not enough to secure selection.
For *Lactococcus lactis* strains (LMG 19460 and IL 1403), the screening of optimal temperature,

medium and agitation suggested different conditions - the MRS culture medium showed a higher specific growth rate and cell density; but maintaining the same temperature (30 °C) and agitation (100 rpm). The use of MRS throughout the transformation, i.e. replacing M17-G in all steps where it is present in protocol 3.2 did not result in the production of transforming colonies. This included change of the solid medium for plating to be MRS agar besides the specific RgM originally used.

MRS is not originally selective but with the lower pH its selectivity towards *Lactobacillus* strains increases. A medium proven effective for bacteriocin production and to favor the growth of *Lactobacillus*, this may be the reason that transformation was not successful for our *Lactococcus* strains.

Lactobacillus strains | The electrotransformation protocol applied without any change did not result in the growth of any transformant colony. In the screening performed so far, we concluded that the best medium for this genus is MRS and the preferred temperature is 37ºC at 0 rpm, and these parameters were changed in the protocol, and attempted to plate colonies both in the regenerating medium and MRS agar, supplemented with 5 µg/mL erythromycin. These changes were not enough to achieve viable transformants with this protocol. Further considerations should be made since the dilutions used for plating were considerably high, of 6 to 9-fold, and only those dilutions were used, meaning that only a small percentage of the successfully transformed bacteria obtained were plated, and from those, the few cells present might be unable to grow into colonies with the antibiotic pressure.

Plasmid purification, quantification and quality assessment | After transformation of LMG 19460 and MG 1363, the two strain that were able to produce transformant colonies, were set to grow in liquid media with selection pressure and used for plasmid purification. After the plasmid DNA was extracted, quantification of the purified pDNA was measured in a nanodrop (Nanovue Plus, GE) and visualized for quality assessment of the plasmid in an Agarose gel electrophoresis.

The concentrations of pTRKH3 obtained in the nanodrop were:

- LMG 19460 (1%) - 100.75 ng/µL
- LMG 19460 (2%) - 132.3 ng/µL
- MG 1363 (1%) – 235.5 ng/µL
- MG 1363 (2%) – 121.7 ng/µL

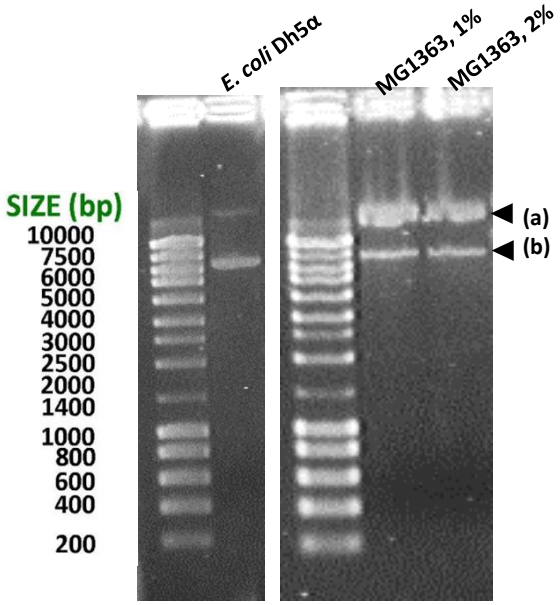

Figure 2 *- Visualization of total pDNA (pTRKH3) in L. lactis* subsp. *cremoris MG1363 cells treated with 1% and 2% glycine by agarose gel electrophoresis alongside the purification of the plasmid from its host cell, E.coli Dh5α.*

Plasmidic DNA can be present as compact supercoiled circular, relaxed open circular and linearized forms. Supercoiled fractions are preferable over open circular and linear fractions, as they are the most biologically active and as transfection procedures are facilitated[14].

The two-band observed for all the purifications correspond to different forms of the plasmid in the cell. The bands are well visible, indicating good quality of pDNA. However, **(a)** seems to represent a high amount of the open-circular structure of pTRKH3, as the band is above the actual size of the plasmid (7766 bp). The band marked as **(b)** appears to slightly set below the 7500 bp mark, which can indicate the plasmid's supercoiled structure and that would be a promising result. Further analysis with a simple digestion of the plasmid DNA should be performed in order to better evaluate plasmid quality in *L. cremoris* MG 1363.

Conclusion and future perspectives

In order to establish a more efficient lactic acid bacteria platform for the production of biopharmaceutical DNA plasmids and recombinant proteins that the one so far developed in LMG 19460, the screening of seven promising LAB strain was mainly focused on their physiological characteristics – Growth optimization and antibiotic susceptibility, and the test of electroporation feasibility under the main goal of obtaining a high yield and high quality pDNA product with less degradation of the produced plasmid.

The screening of different culture media, temperatures and agitation preferences at a bench scale allowed the conclusion that MRS medium is capable of reach higher cell densities when compared to M17 medium supplemented with glucose (20 g/L). This was applied in the protocol for electroporation under the hypothesis that a higher growth rate bacterium will produce more pDNA, the change in protocol from M17-G to MRS had promising outcomes. However, the use of MRS in the electroporation protocol did not show any transformant colonies when plated, either in MRS agar or in the specific regeneration medium. As MRS is a medium developed mainly to boost production of lactic acid and bacteriocins (Novak et al., 1997) bacteria may not be able to manage their resources well when subjected to the electroporation process, as with MRS, LAB growth is corelated to the production of lactic acid and secondary metabolites. However, considerations in the protocol applied should be first adjusted in order to retrieve better conclusions.

Temperature preference in *Lactococcus* strains ranged between 30ºC and 37ºC, and in the presence of agitation (100 rpm), a temperature of 30ºC was considered optimal. For *Lactobacillus* strain, the thermophilic nature of Lb. *plantarum* and Lb. *bulgaricus* **[15]** was demonstrated by their high yields at 40ºC and 43ºC, making them of interest for when the need to apply heat strategies arises. This temperature adaptation was not registered in L. *casei* ATCC 393, which is consistent to the fact that these strains is frequently found in the gut, showing preference for temperatures of 30º, 37ºC.

The LAB strains exhibited a natural high resistance to antibiotics as kanamycin, neomycin, apramycin and spectinomycin, which is not plasmid encoded as all the strains are plasmid-free, reducing the problem of horizontal gene transfer to possible pathogens. For commonly used antibiotics as selection marker, erythromycin, chloramphenicol and ampicillin, all strains showed susceptibility, that allows the use of these antibiotics for selection in the plasmid utilized. Nevertheless, for more traditional applications related to human consumption, this possibility is restrained (Börner et al., 2018) and food-grade solutions should be applied.

Lactococcus lactis subsp. *cremoris* MG1363 was capable to produce higher pDNA concentrations when compared to our reference strain (LMG 19460). Evidence from this work suggests this might be a more suitable candidate, and further screen of its physiological and molecular properties can lead to an efficient gene expression protocol with a high range of applications in the fields of bioengineering. The response to temperature and antibiotic susceptibility of this strain are also encouraging for its use in the future work of this project, as showed the best response to changes in temperature between the *Lactococcus lactis* strains tested, maintaining considerably high growth rates for all temperatures. Regarding antibiotic susceptibility, *L. lactis* subsp. *cremoris* MG1363 might be beneficial for selection approaches with resistance cassettes not only for erythromycin, chloramphenicol and ampicillin – susceptible to all strains tested – but also to apramycin and spectinomycin, which is a relevant update for possibilities in future work.

Although promising results were only obtained for one novel strain in this work, the remaining strains should be studied in more detail, performing some optimizations and adaptations in the electroporation and purification methodologies. The high variability among LAB strains facing growth conditions and stress response might cause the need for a specific optimization study regarding each of the strains of interest. A general optimization protocol for the lactic acid bacteria group might not be the most beneficial work flow to follow in the future, as It will compromise the potentiality of better results for each strain used. Individual study of growth conditions is necessary prior to the application of these to transformation protocols and other biotechnology strategies in any novel LAB strain

These results highlight the possibility to establish, after several steps of optimization, Lactic Acid Bacteria as suitable and safer host to produce pharmaceutical-grade pDNA, highlighting the laboratory strain *Lactococcus lactis* subsp. *cremoris* MG1363, to be used in DNA vaccination

taking benefits of their GRAS status, their probiotics characteristics and the absence of lipopolysaccharides in their membranes.

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I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the University of Lisbon.

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