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# 1. Introduction

## 1.1. Lactic acid bacteria

Lactic acid bacteria (LAB) belong basically to the order *Lactobacillales*. The genera harboring LAB comprise *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Bifidobacterium*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, *Lactosphaera*, *Bacillus*, *Halolactobacillus*, *Carnobacterium*, *Vagococcus* and many more. Lactic acid bacteria constitute a heterogenous group founded on metabolic characteristics, including temperature range of growth and agglutination, fermentation profiles and metabolic pathways. Nowadays identification of LAB species is often based on 16S RNA sequencing which is not sufficient to yield a reliable and proper result. Trustworthy classification has to be based on both genotypic and phenotypic methods. (Pot and Tsakalidou, 2009) In accordance, Rossella-Mora and Amann (2001) claim that besides genetic evidence, phenotypic properties are required to discriminate a species from its closest phylogenetic neighbour.

Traditionally LAB are grouped into obligatory homofermentative, facultatively heterofermentative and obligatory heterofermentative bacteria. They are usually catalase-negative, non-motile and non-spore forming cocci, coccobacilli or rods. All LAB are defined by the ability to produce considerable amounts of lactic acid as a major end product of carbohydrate metabolism. Lactic acid bacteria are also gram-positive and acid-tolerant; they range from aero-tolerant to anaerobic and have in general a low GC-content. By acidifying their medium, they inhibit growth of other bacteria, which is a desirable feature used in food preservation. (Hammes and Vogel, 1995)

Representatives are usually found in nutrient-rich environments - soil, sewage, silage, the human oral cavity, the intestinal tract, the vagina and many more habitats rich in food substrates or plant materials - basing their metabolism on complex nutrients. Especially industrial lactobacilli used for decades for milk or meat fermentation are often referred to as “being crippled in metabolism”. Due to their limited biosynthetic capacity they require vitamins (folic acid, vitamin B<sub>12</sub>, pantoic acid and more), amino acids, salts, fatty acid esters and nucleic acid derivatives for growth (Kandler, 1986). There are exceptions like *L. plantarum* which is able to prosper in a variety of niches and will be described later in more detail.

LAB are used in food- and feed-fermentations, like production of vegetable, meat and dairy products, but also play a significant role in preservation of ensiled forages. Commercial starter-cultures are available to ensure steady and reproducible acidity, flavour, texture and colour of the end-product or to prevent uncontrolled fermentation processes. They are not only used for acidification and fermentation of sugars or flavour compounds, but also for their proteolytic and lipolytic activities. Products of citrate fermentation influence aroma and quality of fermented goods (Pot and Tsakalidou, 2009).

### 1.1.1. LAB used in the study

#### 1.1.1.1. *L. plantarum*

*Lactobacillus plantarum* is a facultatively heterofermentative bacterium that produces DL-lactate, and contains *meso*-diaminopimelic acid in its cell wall. It is closely related to *L. paraplantarum*, *L. pentosus*, and *L. arizonensis* and cannot be distinguished from these species by 16S rRNA sequencing only (Hammes and Hertel, 2003). Molecular techniques to reliably identify *L. plantarum* are at hand, but often neglected, which might result in industrial products – like probiotics - with false descriptions. This problem is not exclusive to *L. plantarum* as will be discussed later.

*L. plantarum* is encountered in many niches like dairy, meat, and vegetable or plant fermentations, including corn and grass silages. It was initially claimed to be a natural inhabitant of the human gastrointestinal tract and therefore found its way in probiotic products. The genome sequence of *L. plantarum* is known and one of the largest, providing a basis for its broad metabolic capacity. In effect, the strain WCFS1 was sequenced and thoroughly tested. It was found to have a high number of sugar import systems and the capacity to use a large variety of carbon sources which affirms the high versatility and ecological flexibility of the species. It also displays a considerable number of genes coding for surface-anchored proteins which enables the strain to potentially bind a high number of substrates or surfaces. (Kleerebezem *et al.*, 2003)

In addition, *L. plantarum* has a repertoire of stress response systems, namely a heat-shock response, a cold-shock response, an acid-stress system, alkaline-

shock proteins, oxidative stress-related proteins, and osmoprotection, which enhances its ability to thrive in different environments (Tamime, 2005).

#### ***1.1.1.2. L. buchneri***

*Lactobacillus buchneri* is commonly used in corn and grass silages (Driehuis *et al.*, 1999; Li and Nishino, 2011). It increases stability of silages against yeast and moulds when exposed to air mainly by driving acetic acid production (Driehuis *et al.*, 2001; Holzer *et al.*, 2003). This is due to its ability to produce 1,2- and 1,3-propanediol from glycerol while metabolizing glucose or fructose. The co-fermentation results eventually in re-oxidation of the originally produced lactate to acetate which is deterrent to a number of other microorganisms. (Cunha and Foster, 1992)

The heterofermentative bacterium contains lysine-D-asparagine in its cell wall (Pot and Tsakalidou, 2009).

#### ***1.1.1.3. L. reuteri***

A particularly important strain in probiotics and this present work is *Lactobacillus reuteri*, a heterofermentative lactobacillum that is used in animal nutrition, yoghurt products and pharmaceutical preparations. *L. reuteri* can also be found in sourdough populations (Dal Bello *et al.*, 2005).

It is characterized by the ability to produce a bacteriocin called reuterin and therefore has the potential to inhibit growth of pathogenic bacteria like *E.coli* and *L. monocytogenes* (Klein *et al.*, 1998; El-Ziney and Debevere, 1998). Furthermore, it has been identified as an indigenous inhabitant of the gastrointestinal tract of human, pig and chicken exhibiting tolerance to bile salts and acid as well as adhesion to the intestinal epithelium (Liu *et al.*, 2005). There is significant effort to exploit production of galacto-oligosaccharides (GOS) by *L. reuteri* for pre- and synbiotic products (Splechtna *et al.*, 2006). As will be described later in more detail  $\beta$ -galactosidases are able to catalyze transgalactosylation reactions resulting in GOS which have a beneficial influence on the intestinal probiotic microflora.

Orally administered *L. reuteri* has also been found to exhibit immunoadjuvant activity by increasing expression of inflammatory cytokines and could therefore be used as a potential delivery vector for therapeutic proteins (Maassen *et al.*, 2000).

#### 1.1.1.4. *E. faecium*

The facultatively anaerobic and coccoid *Enterococcus faecium* occurs chiefly in pairs or short chains, the peptidoglycan type is Lys-D-Asp (Schleifer and Klipper-Bälz, 1984).

Strains of enterococci are regularly used in animal nutrition, mainly pig and poultry. For instance, *Enterococcus faecium* is a common animal probiotic. It is also often isolated from clinical material known to cause nosocomial infections in immunocompromised hosts. (Klein *et al.*, 1998)

*E. faecium* are also commonly used in corn and grass silages (Driehuis *et al.*, 1999; Li and Nishino, 2011).

#### 1.1.1.5. *L. lactis*

*Lactococcus lactis* is used as a dairy starter culture, and has more recently been studied as a live vehicle for the production of therapeutic molecules (Bahey-El-Din and Gahan, 2010). It is known to produce significant amounts of lactic acid via homolactic fermentation and small amounts of flavour compounds (Tamime, 2005). It is able to pass the gastrointestinal tract of mice, but is not able to establish as a natural inhabitant (Hiromi *et al.*, 2003).

The first strain to be sequenced was IL1403 (Bolotin *et al.*, 2005) which was obtained by plasmid-curing. It was found that many traits were actually encoded by plasmids including genes for lactose metabolism and degradation of casein. It was also hypothesized that the strain as having a long history in dairy production had lost several biosynthetic capabilities compared to strains prospering in plant or animal environments. (Tamime, 2005)

## 1.2. Probiotics

Lactic acid bacteria are traditionally used in food industry and silage, but since the 1990s their scope of applications includes a new field, namely probiotics.

In 2002, Holzapfel and Schillinger defined probiotics as a term referring to “viable organisms that promote or support a beneficial balance of the autochthonous



microbial population of the gastrointestinal tract". A more general definition was given by Tannock (2002) and Guarner et al (1998) who spoke of "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition".

In either case it is crucial to understand the composition and characteristics of the enteric microflora. The mucosal surface of the gastrointestinal tract is sterile in the case of new-borns, but is quickly colonized by numerous genera. Depending on diet, medication, stress, age, genetic background and physiological state of the host, the exact composition of bacteria will be unique for each human (Tannock, 1997). Furthermore, the gastrointestinal tract (GIT) itself constitutes a spatially heterogeneous environment. The stomach and the first two-third of the small bowel are difficult to colonize due to the highly acidic pH of the stomach (up to pH 2) and toxic bile salts (Tannock, 1995). Hence, nutrient availability decreases in distal sites while bacterial numbers increase. Unique ecological niches are generated which eventually – according to the niche exclusion theory – support only one type of well-adapted microbe each (Hardin, 1960). Allochthonous bacteria, introduced by food or residing in the oral cavity, will therefore face competitive exclusion as they pass the gastrointestinal tract that is already occupied by autochthonous bacteria or true residents.

According to Gibson (1995) and Rastall (2004), the enteric microflora is dominated by strict anaerobes, like clostridia, peptococci, *Bacteroides* spp., *Bifidobacterium* spp. and *Atopobium* spp.. Studies using anaerobic culturing techniques (Dal Bello et al, 2003) and culture-independent fluorescent in situ hybridization (Harmsen et al., 2002) as well as GIT biopsy samples (Eckburg *et al.*, 2005) suggest that only few *Lactobacillus* species inhabit the human gastrointestinal tract and are more commonly present as allochthonous members. In 2001, Reuter identified *L. ruminis*, *L. reuteri* and *L. gasseri* as the only true autochthonous lactobacilli of the human gastrointestinal tract.

Several aero-tolerant lactobacilli are currently used in a considerable number of probiotic products, as they are well known and easy to handle based on a long history of food fermentation. In addition, they are thought to exert health benefits by antioxidant activity, production of bacteriocins that are active against pathogens, suppression of *H. pylori* colonization and infection, reduction of antibiotic associated colitis, and production of anti-mutagens and binding of mutagens (Ljungh and

Wadstrom, 2006). Also alleviation of lactose intolerance and cholesterol lowering effects have been linked to probiotics (Holzapfel and Schillinger, 2002).

The putative ability to modulate the gastrointestinal flora and beneficially influence health has led to a huge economic interest in probiotic strains, favouring lactobacilli that can be handled and produced industrially in an easy way (O'Sullivan *et al.*, 1992; usprobiotics.org).

However, most of these strains have been shown to be autochthonous to the human oral cavity, but only transient passengers to the GIT (Kimura *et al.*, 1997). Reuter (2001) also claimed that "descriptions of strains used by producers of probiotic cultures are often incomplete or even false". He criticizes industrial mismanagement which results in random selection of strains, unviable probiotics and deception of consumers. Persistence of lactobacilli in the GIT after oral administration has ceased also remains questionable when transient strains are used. Nonetheless, colonization might not be essential for a successful probiotic. Allochthonous strains might as well act by stimulating the immune system. Even dead cells were reported to exhibit beneficial immunological effects (Mottet and Michetti, 2005).

Classification of autochthonous lactobacilli was also performed in pigs, chicken and rodents. These animal species display large numbers of truly inhabitant lactobacilli in the proximal gut. Their stomachs are mainly characterized by non-secretory epithelium which enables adherence of lactobacilli and formation of biofilms accomplished by production of extracellular polymeric substances. (Tannock, 1992) Such stratified squamous epithelia are not present in the human gut, but in the oral cavity and vagina, habitats with high numbers of lactobacilli (Walter, 2008).

In 2002, Leser *et al.* identified *L. reuteri* as an autochthonous member of the rodent and porcine gut. Further studies examined crucial proteins generated by *L. reuteri* that enable adherence to the gastrointestinal epithelium, cell aggregation, formation of biofilms and resistance to low pH values in mice, prerequisites for the existence of stable populations (Walter *et al.*, 2008). *L. reuteri* was also shown to prevent colitis in immunodeficient mice (Madsen *et al.*, 1999).

In human studies, *L. reuteri* was shown to suppress *H. pylori* infections and to ameliorate acute diarrhoea in young children (Shornikova *et al.*, 1997; Imase *et al.*, 2007). Various human isolates were shown to produce reuterin, an antimicrobial compound that is thought to model and shape the composition and spatial

architecture of the gastrointestinal microbiota (Jones and Versalovic, 2009). Furthermore, it is interesting to note, that *L. reuteri* strains in this study could be divided into immunosuppressive and immunostimulatory strains. Product description on species level does not offer sufficient information on origin, autochthony and suitability as a probiotic.

Once again it has to be pointed out that effectiveness of probiotics is always strain-specific. Each strain has to be examined on its own to prove beneficial effects and to understand the underlying mechanisms.

### 1.3. Prebiotics and synbiotics

Prebiotics are growth-promoting substrates, mainly carbohydrates that resist digestion in the small intestine. After reaching the large intestine, they are fermented by the microflora and support a favourable enteric microbial balance. Roberfroid (2007) defined prebiotics as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microflora that confers benefits upon host well-being and health”. It is vital to supply probiotic bacteria with fermentable carbohydrates as many of their beneficial effects rely on their metabolic activities. This includes the activity of enzymes like glucuronidase, glucosidase and nitroreductase as well as end products of fermented fatty acids, amino acids, sterols, and mucins.

Roberfroid claimed three criteria to be fulfilled for classification of prebiotics:

- resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption,
- fermentation by intestinal microflora, and
- selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being.

Furthermore, Roberfroid grouped prebiotics in two main categories: inulin-type prebiotics, which include fructooligosaccharides, oligofructose and inulin, and galactooligosaccharides (GOS). These complex carbohydrates are neither absorbed by the host nor utilised by the majority of gram-negative bacteria (Collins *et al.*, 1998). Further prebiotic candidates exist, like glucooligosaccharides, isomalto-oligosaccharides, soybean oligosaccharides, xylo-oligosaccharides, gentio-oligosaccharides, lactosucrose, polydextrose, but have to be examined in more detail to prove their eligibility.

Inulin-type prebiotics have been studied extensively. In 2010, Patterson *et al.* conducted a study on inulin supplemented diet on young pigs. Inulin is a general term comprising  $\beta$ -fructans of varying chain lengths. Although it is not hydrolysed by mammalian digestive enzymes, it becomes significantly degraded in the ileum - mainly fermented by bifidobacteria. Patterson demonstrated in experiments with young pigs that upon ingestion of inulin populations of beneficial bifidobacteria and lactobacilli were enhanced with reductions in less desirable bacteria as *Clostridium* spp. and that iron status of prebiotic-fed animals improved. They also demonstrated downregulation of inflammation-related genes in the colonic mucosa. Others demonstrated a beneficial influence of inulin-type prebiotics on mineral absorption in human and animal models (Griffin *et al.*, 2003; Ohta *et al.*, 1995).

GOS are derived from lactose by transgalactosylation, where any sugar molecule can be used as a nucleophile accepting the galactosyl. Most bifidobacteria and lactobacilli are able to metabolize GOS that serve as nutrients for themselves and other probiotic bacteria (Saulnier *et al.*, 2007). Their numbers increase, while pathogenic bacteria, like *Bacteroides* decrease upon ingestion of these oligosaccharides (Ito *et al.*, 1993). *L. reuteri* has both a  $\alpha$ - and  $\beta$ -galactosidase that is able to transfer galactose from lactose to other lactose molecules, building galactooligosaccharides. Especially the  $\beta$ -galactosidase of *L. reuteri* has been subject to intensive research and genetic engineering with the aim to increase production of GOS. The oligosaccharides could be used as plain prebiotic products, in combination with probiotic products or even genetically altered *L. reuteri* could be administered as a whole to poultry, pig and more.

GOS increase the production of short chain fatty acids in the colon, hence facilitating calcium and magnesium absorption and energy supply to the colonic epithelium in humans (Sako *et al.*, 1999). Tzortzis *et al.* (2005) investigated the prebiotic effect of GOS in weaned pigs; they reported reduced pH in the proximal colon, increased amounts of short chain fatty acids and reduced attachment of enteropathogenic *E. coli* and *Salmonella enterica*. The concept is that GOS might competitively bind bacteria and prevent attachment to the gut epithelium by acting as a functional mimic for bacterial receptors. The tested GOS mixture was also shown to selectively raise bifidobacterial populations.

Prebiotics can be incorporated in several probiotic products, supporting the viability of the targeted probiotic strain; such a combination is termed a synbiotic.

These compoundings have to be tested and well matched to ensure optimal boost of desired probiotic strains.

The concomitant administration of *L. reuteri* and its own oligosaccharides was shown to enhance specifically growth of the strain itself, but also bifidobacteria (Rastall, 2004). A study combining *L. reuteri* with soybean oligosaccharides resulted in higher resistance of *L. reuteri* to bile salts and increased bioavailability of isoflavones (De Boever *et al.*, 2001).

A future aim is to design species-specific prebiotics and therefore to ensure growth of targeted microbial populations in the gastrointestinal tract, producing tailored synbiotics for specific health attributes.

Pro- and prebiotics should be administered thoughtfully, as dose-related adverse effects like flatus, bloating, abdominal cramps and diarrhoea are possible due to excessive fermentation or osmotic potential of certain carbohydrates (Holzapfel and Schillinger, 2002).

#### **1.4. Genetic engineering of lactic acid bacteria**

Lactic acid bacteria are used to produce proteins for the food industry, metabolites, enzymes and proteins of therapeutic value including antigens, cytokines, and pro- and prebiotics. This is mainly achieved by genetic engineering which allows for introduction of foreign genes, modulation of gene expression, and efficient secretion (De Mey *et al.*, 2010). Genetic engineering is therefore aimed to be improved constantly.

Metabolic processes like production of GOS by *L. plantarum* and *L. reuteri* can be enhanced by introducing strong constitutive promoters upstream of genes coding for the relevant enzymes (De Mey *et al.*, 2010). Production of GOS would therefore occur exactly at the site where probiotic bacteria reside. The promoter of lactate dehydrogenase is one possible candidate for overexpression purposes. LDH is a highly active enzyme needed to relieve the cell constantly of accumulated NADH and to regenerate NAD<sup>+</sup>.

Up to date several tools for genetic engineering have been successfully tested and established. They are crucial for protein production in dairy and food industry. Efficient expression vectors have already been developed for *Lactococcus* (Wells *et al.*, 1993) where expression of recombinant proteins is ensured by constitutive promoters or inducible systems. The most common controllable expression systems

are based on either lactose-inducible transcription of the *lac* operon or on the food-grade inducer nisin. Nisin is a bacteriocin which was discovered in *Lactococcus lactis*. It induces transcription of the *nisA* gene via signal transduction mediated by a histidine kinase and a response regulator. (Kuipers *et al.*, 1995) The nisin based system is suitable for food-grade and controlled overproduction of desired proteins. Strains have to be chosen that are known for their efficient secretion of recombinant proteins for industrial protein production. The capacity to secrete heterologous proteins varies from species to species. Hols *et al.* (1997) demonstrated the ability of *L. plantarum* to secrete up to 10 mg/L of a specific antigenic fusion protein.

It is advisable to use intra-species promoters if the recombinant organism itself is administered to avoid adverse effects and to ensure safety of the final customer. Furthermore, it is absolutely necessary to work with GRAS bacteria only to avoid health risks otherwise imposed on the patient. Further prerequisites are tolerance to low pH and bile salts if the gut is the designated destination.

A crucial advantage of LAB and their applications is that most lactic acid bacteria have GRAS status (= generally regarded as safe), which means that they have no recorded toxic or pathogenic activity. Nevertheless, occasionally there are case reports on systemic infections caused by LAB in immunocompromised hosts. For instance, *L. rhamnosus* was identified as causative agent in endocarditis (Mackay *et al.*, 1998; Sipsas *et al.*, 2002). Also enterococcus should be avoided as a model for live vectors, as multi-resistant enterococci are able to transfer resistance plasmids to *S. aureus* (Noble *et al.*, 1992).

Regarding the high standards of security it would be advantageous to succeed in genetic engineering and transformation of *L. reuteri*. Others have expressed the  $\beta$ -galactosidase from *L. reuteri* in *E. coli* leading the way to industrial production of galacto-oligosaccharides (Nguyen *et al.*, 2007). The  $\beta$ -galactosidase could also be overexpressed in yeast or other food-grade systems yielding GOS for pre- or synbiotic products, but administration of live recombinant probiotics needs intraspecies genetic engineering, i.e. enhancing the  $\beta$ -galactosidase gene by introducing a promoter of a household gene of *L. reuteri* into *L. reuteri*.

One prospering field – as an example - based on genetic engineering is metabolic engineering. The main outcome of fermentation in lactic acid bacteria can be shifted to acetic acid, diacetyl, acetaldehyde, and ethanol. These by-products can be accumulated by disruption or overexpression of metabolic genes. Disruption of the

ldh-gene in *L. plantarum* and *L. lactis* inhibited further production of lactic acid and resulted in mixed acid fermentation (Platteeuw *et al.*, 1995; Ferain *et al.*, 1998). Additionally, cloning of ethanol genes, coding for alcohol dehydrogenase and pyruvate decarboxylase, derived from an efficient ethanol-producing bacterium in LDH-negative lactobacilli highly improved ethanol yields. *L. lactis* engineering was also used successfully to increase production of the butter aroma compound diacetyl and the amino acid alanine (Hugenholtz and Kleerebezem, 1999; Hols *et al.*, 1999), while metabolic engineering in *Lactobacillus fermentum* aimed at producing high levels of mannitol, L-lactic acid and pyruvate, needed in large quantities for the synthesis of biopolymers and pharmaceuticals (Li *et al.*, 2001).

## 1.5. Electrotransformation

### 1.5.1. General aspects of electrotransformation

Electroporation is a method aiming to introduce DNA into foreign cells and therefore a crucial tool in genetic engineering. An electric field is applied to a mixture of plasmids and electrocompetent cells to stimulate DNA uptake. Although the method is widely used, the molecular process itself remains unknown.

Xie *et al.* (1990) worked on *E. coli* and proposed that DNA is absorbed on the cell surface and transfers into the cell after formation of membrane pores by induction of an overcritical membrane potential during the electric pulse. Required field intensities are either reached by appliance of high voltage or usage of narrow electroporation cuvettes. After membrane pores are formed, the negatively charged DNA is supposed to move into the cells in an electrophoresis-like manner.

Xie *et al.* (1990) hypothesized that the membrane might take an active role in plasmid absorption. Extracellular structures, like exopolysaccharides, S-layers and pili, are thought to be an obstacle to plasmid interaction and efficient electroporation, as well as thick cell-walls that might be more difficult to penetrate. Parameters, like choice of growth medium, growth temperature, treatments affecting cell wall organization, and more, have to be optimized, as they differ extremely from species to species.

Electrical parameters, like pulse duration, field strengths, resistance of the sample, and many more have a crucial influence on successful penetration. A

prerequisite of effective electrotransformation is to apply high field intensities to bacterial solutions. This can be achieved either by narrowing the width of electroporation cuvettes or increasing the applied voltage. The voltage across the electrodes rises to a peak voltage and declines in an exponential waveform. A built-in resistor determines the decay time of the field applied on the sample (Eynard and Teissié, 2000). The time constant which expresses pulse length is usually set at 5 ms. Lower constants may indicate a failure in pulse delivery and are therefore monitored in every experiment.

Thorough washing of the sample is important as not to increase the conductivity by residual ions, to keep resistance high, and to avoid arcing.

Gram-negative bacteria are usually the easiest ones to electroporate. In many cases, transformants can be obtained by simply removing ions and salts through two or three washing steps. Thorough washing is important to avoid arcing; otherwise the charge is conducted through the medium causing severe damage to the electroporated cells and loss of viability.

In a last step, cells are usually washed and resuspended in an electroporation solution like aqueous glycerine or PEG - lowering the conductivity of the sample - before applying a pulse. Electroporation itself is carried out at ice-cold temperatures to avoid heat damage to the cells. During the pulse the electric energy is converted into heat, increasing the temperature of the sample.

It is also advisable to incubate cells with plasmids before electroporation to increase contact and absorption of plasmids to the cell surface. Pre-incubation at low temperatures might additionally inhibit extracellular nucleases and degradation of plasmid DNA.

### **1.5.2. Electrotransformation of LAB**

Electrotransformation of gram positive LAB usually requires additional steps to transcend the rigid cell wall. A panoply of cell pre-treatments has already been proposed to alter cell wall structure of refractory gram-positive strains.

Glycine is added to the growth medium to replace D-alanine of peptidoglycan precursors and to reduce cross-linking by being a poor substrate for transpeptidation (Dunny *et al.*, 1991); threonine inhibits diaminopimelic acid incorporation in peptidoglycan (McDonald *et al.*, 1995), while treatment with lysozyme removes partially the cell wall (Powell *et al.*, 1988). Haynes and Britz (1989) reported that



Tween80, an emulsifier known to prevent clumping of mycobacteria, changes the mycolic acid composition of corynebacteria, Palomino *et al.* (2010) proposed the use of NaCl as a cell-weakening agent for *Lactobacillus* transformation. In 2007, Papagianni *et al.* described successful transformation of *Lactococcus lactis* using lithium-acetate and DTT, which are common in yeast transformation.

The milestones in establishing methods for electroporation of LAB include growth in glycine and sucrose (Holo and Nes, 1989), the use of PEG as electroporation or storage solution (Aukrust and Blom, 1992), and thorough testing of suitable electrical parameters (Axelsson and Ahrne, 1990).

Nevertheless parameters, like choice of cell-wall weakeners or electrical settings, depend on the strain. Even within the same species the susceptibility to electrotransformation procedures and physiological needs are significantly diverse. Every strain itself has to be examined thoroughly to develop an operative electrotransformation protocol.

## 2. Objective

One aim of this work was to compare the effectiveness of selected published electrotransformation protocols for our industrial LAB strains derived from the genera *Lactobacillus*, *Lactococcus* and *Enterococcus*. Electrotransformation is a prerequisite for effective genetic engineering in industrial applications.

We paid special attention to the development of an electroporation method for a specific industrial *L. reuteri* strain by examining growth media, washing solutions and electrical parameters.

### 3. Materials and Methods

#### 3.1. Materials

##### 3.1.1. Microorganisms

###### Lactobacillaceae

*Lactobacillus plantarum* CD032

*Lactobacillus plantarum* CD033

*Lactobacillus buchneri* CD034

*Lactobacillus buchneri* CD035

*Lactobacillus reuteri* CD037

*Lactobacillus reuteri* DSM 20016

###### Enterococcaceae

*Enterococcus faecium* CD036

###### Streptococcaceae

*Lactococcus lactis* MG1363

###### Enterobacteriaceae

*Escherichia coli* JM109

##### 3.1.2. Equipment

Table1. Equipment

Supplier	Device
SCIE-PLAS (UK), Beckman Coulter (USA)	agarose gel units (Midi Horizontal Gel Unit, Avanti J-20-XP)
Eppendorf (Germany), Jouan (Germany)	centrifuges (5415 D, C312)
Heraeus (Germany)	class II laminar flow (Herasafe)
Hofer (USA)	gel caster (260 series)
Bio-Rad (USA)	gel documentation system (GelDoc 2000)
WTB-Binder (Germany), Heraeus (Germany)	incubators (B 115, BK 6160)

Supplier	Device
WTW (Germany)	pH meter (pH 521)
Sartorius (Germany), Mettler-Toledo (Germany)	scales (A-120-S, SM-L)
Implen (Germany)	NanoPhotometer
BTX Harvard apparatus (USA)	electroporator (ECM 630 Precision Pulse)
UVP (USA)	transilluminator Benchtop 3UV
peqLAB (Germany)	spectrophotometer (NanoDrop1000)
Greiner Bio-One (Germany)	pipette tips, centrifuge tubes

### 3.1.3. Reagents

Table 2. Reagents

Supplier	Reagents
Roth (Germany)	Tris, DMSO, EDTA, MRS agar and bouillon, salts, sugars, peptone, agar agar
Sigma (Germany)	Tween80, ethidiumbromide, ammonium citrate, glycine, sodium acetate, HEPES
Invitrogen (USA)	agarose
Oxoid (UK)	M17 broth and agar
Riedel-de Haen (Germany)	MnSO <sub>4</sub>
Fluka (Switzerland)	meat extract, MgSO <sub>4</sub> , yeast extract

### 3.1.4. Growth media

Media were prepared with deionized water and sterilized by autoclaving at 120 °C for 20 minutes. Sensitive reagents like glycine or antibiotics were sterile-filtered.

#### *Growth media for LAB*

##### MRS Bouillon

10 g/L peptone

4 g/L yeast extract

8 g/L meat extract  
2 g/L di-potassium hydrogen phosphate anhydrous  
5 g/L sodium acetate  
2 g/L ammonium citrate tribasic anhydrous  
0.4 g/L magnesium sulfate heptahydrate  
0.056 g/L manganese(II) sulfate-1-hydrate  
1 g/L Tween 80  
20 g/L glucose

Maillard reaction during autoclaving resulted in dark-coloured MRS.  
Glucose was autoclaved separately, where light-coloured MRS Bouillon was needed.

#### MRS agar

MRS bouillon was supplemented with 10 g/L agar agar before autoclaving.

#### M17 broth

5 g/L tryptone  
5 g/L soy peptone  
5 g/L "Lab-Lemco" powder  
2.5 g/L yeast extract  
0.5 g/L ascorbic acid  
0.25 g/L MgSO<sub>4</sub>  
19.0 g/L di-sodium-glycerophosphate  
Glucose and lactose were added after separate autoclavation.

### ***Growth media for E. coli***

#### LB medium

peptone 10 g/L  
yeast extract 5 g/L  
sodium chloride 10 g/L

#### LB agar

LB medium was supplemented with 15 g/L agar agar before autoclaving.

### 3.1.5. Antibiotics

Ampicillin and Chloramphenicol were purchased from Roth (Germany). Ampicillin was used for *E. coli* in LB medium with a concentration of 100 µg/mL. Chloramphenicol was used both for *E. coli* and LAB in different concentrations. MRS was supplemented with 5 µg/mL chloramphenicol , LB with 25 µg/mL.

Ampicillin

stock solution: 100 mg/ml (in ddH<sub>2</sub>O)

Chloramphenicol

stock solution: 25mg/ml (in EtOH)

### 3.1.6. Enzymes

KOD polymerase was purchased from Novagen (USA), HybriPol from Bioline (UK)

### 3.1.7. Primers

Primers for colony PCR

pUC19\_for

5'-AGTTGCGCAGCCTGAATGG-3'

CATseq\_back

5'-GTTATTGGGATAAGTTAGAGC-3'

### 3.1.8. Plasmids

The vectors are based on pUC19 containing a chloramphenicol acetyl transferase (CAT) gene from pC194 for selection in LAB. Each one has an additional ori to improve replication in LAB. pCDWV01 is a minimal version of pCDW01 lacking the pMB1 ori and the ampicillin resistance gene. (Leenhouts et al., 1991; Kiewiet et al., 1993; Sørvig et al., 2005; Gryczan et al., 1982.)

Table 3. Plasmids used in the study.

plasmid	donor plasmid of ori	replication	donor organism
pCDW01	pWV01	rolling circle	<i>Lactococcus lactis</i>

pCDWV02	pWV02	theta replication	<i>Lactococcus lactis</i>
pCD256	p256	rolling circle	<i>L. plantarum</i>
pCDE194	pE194	rolling circle	<i>B. subtilis</i>

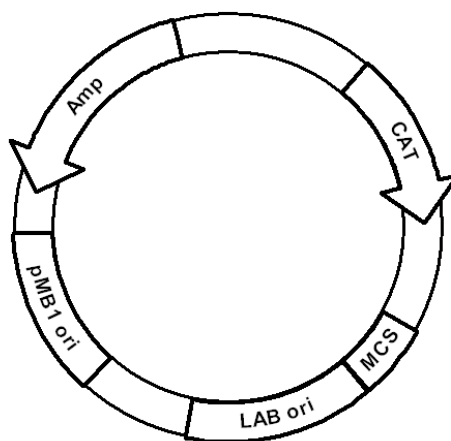


Figure 1. Map of the pCD vectors carrying an origin of replication from either *Lactococcus lactis*, *L. plantarum* or *B. subtilis* (LAB ori) designated for replication in lactobacilli, an origin of replication for optimised replication in *E. coli* (pMB01 ori), a multiple cloning site and two antibiotic resistance genes.

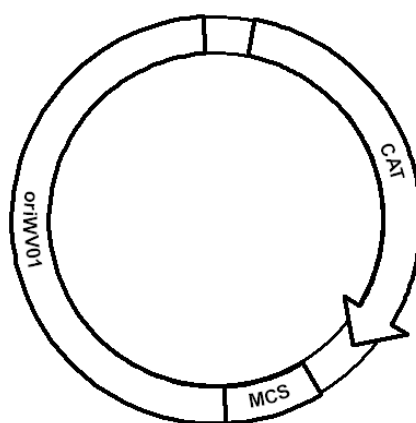


Figure 2. Map of pCDWV01, derived from pCDW01, containing a *Lactococcus* ori, a multiple cloning site and a chloramphenicol resistance gene.

## 3.2. Methods

### 3.2.1. Growth conditions

Strains were grown – unless otherwise stated – under following conditions.

Table 4. Growth conditions of LAB strains.

Strain	Temperature (°C)	Medium	Oxygen
<i>L. plantarum</i> CD032	30	MRS bouillon	no
<i>L. plantarum</i> CD033	30	MRS bouillon	no
<i>L. buchneri</i> CD034	37	light MRS bouillon	yes
<i>L. buchneri</i> CD035	37	MRS bouillon	yes
<i>E. faecium</i> CD036	37	MRS bouillon	yes
<i>L. reuteri</i> CD037	37	light MRS bouillon	no
<i>L. lactis</i> MG1363	30	M17	no
<i>E. coli</i> JM109	37	LB	yes

### 3.2.2. Preparation of electrocompetent cells and electroporation

#### *Initial protocol combined with glycine*

(Axelsson and Ahrne, 1990)

#### *L. plantarum* CD032 and CD033

*L. plantarum* CD032 and CD033 were incubated at 30°C without aeration. The cultures were diluted to an OD<sub>600</sub> of 0.2 in MRS supplemented with 1% glycine. The cells were harvested at an optical density of approximately OD<sub>600</sub> 0.6. Centrifugation was performed at 10, 000 rcf for 10 minutes to pellet the cells. They were washed three times with dH<sub>2</sub>O, once with 30 % PEG<sub>1500</sub> and suspended in fresh 30 % PEG<sub>1500</sub> (4 mL). Centrifugation were increased in speed and time when necessary. 40 µL aliquots of resuspended cells were either frozen in liquid nitrogen and stored at – 80 °C or used immediately. OD<sub>600</sub> of the aliquoted cells was between 11 and 19.

Thawed or freshly prepared aliquots were held 10 min on ice after mixing 1 µg of plasmid with each aliquot. The cell solution was transferred into precooled electroporation cuvettes; during pipetting trapping of air bubbles was avoided. A



pulse of 2000 V/200  $\Omega$ /25  $\mu$ F was applied to both *L. plantarum* strains before incubation in 360  $\mu$ L MRS at 30°C for 2 hours. Undiluted and serial dilutions of recovered cell suspensions were spread and incubated on either MRS chloramphenicol agar or plain MRS agar. For evaluation of cell survival and transformations efficiency it is further necessary to spread cell suspensions before the appliance of an electric pulse. Transformant colonies are visible after two days in general.

#### *L. buchneri* CD034

An overnight culture of *L. buchneri* CD034 in MRS was diluted to an OD<sub>600</sub> = 0.2 and supplemented with 1% or 2% glycine. Cells were incubated at 37 °C aerobically and harvested at 0.4 OD<sub>600</sub>. Washing and pre-pulse treatment were carried out as above. The electrical parameters were set at 2,500 V and 200  $\Omega$  (25  $\mu$ F). *L. buchneri* CD034 was incubated post-pulse 2 hours in MRS containing 0.5 M sucrose at 37 °C with agitation and plated on MRS agar with chloramphenicol.

#### *L. buchneri* CD035

*L. buchneri* CD035 was incubated at 37 °C overnight in MRS under oxidative conditions. The cells were diluted to OD<sub>600</sub> = 0.2 in MRS 1% glycine and harvested at 0.4 to 0.5 OD<sub>600</sub>. Washing and aliquotation were performed as described above. Electrical parameters of 2,500 V/200  $\Omega$  (25  $\mu$ F) were applied to *L. buchneri* CD035 and cells were incubated in MRS 0.5 M sucrose at 400 rpm at 37 °C for 2 hours before plating on MRS chloramphenicol plates.

#### ***Lithium-acetate method***

Overnight cultures were grown under strain-specific conditions (see 3.2.1) and diluted to an OD<sub>600</sub> of 0.2. Cells were harvested after reaching an OD<sub>600</sub> of 0.4 – 0.6. Cells were centrifuged for 15 min and resuspended in 30 mL lithium acetate solution (0.6 M sucrose, 100 mM lithium-acetate, 10 mM Tris-HCl, pH 7.5). 120 mL of lithium acetate-DTT (0.6 M sucrose, 100 mM lithium-acetate, 10 mM Tris-HCl, pH 7.5, 10 mM DTT) solution were added and the cell solution was incubated for 30 min at room

temperature and 50 rpm. The suspension was washed twice with ice-cold 0.3 M sucrose, once with 25 mL 50 mM EDTA and again twice with 0.3 M sucrose. The cells were resuspended in 0.3 M sucrose. Electrical parameters were set as described for the initial protocol.

#### ***High efficiency protocol for *Lactococcus lactis* (Papagianni M. et al, 2007)***

Overnight cultures of *L. lactis* were grown in M17 supplemented with 0.5 % glucose and 40 mM threonine and diluted 1:12.5 in 25 mL of M17 with glucose. Cells were harvested at OD<sub>600</sub> ~ 0.3 and suspended at room temperature for 30 minutes in 8 mL of 100 mM LiAc, 10 mM DTT, 0.6 M sucrose, and 10 mM Tris-HCl, pH 7.5. Following pretreatment, cells were washed twice with ice-cold ddH<sub>2</sub>O, once with 50 mM EDTA, ddH<sub>2</sub>O and twice with 0.3 M sucrose. Cells were immediately electroporated by a single pulse at 2.5 kV, 200 Ω and 25 μF using 1 μg of DNA. The cell suspension was diluted in 5 mL M17 containing glucose and 1% sucrose and incubated for 2 hours at 30 °C.

#### ***High salt protocol***

(Palomino et al., 2010)

Cells were cultured for 21 hours at 37 °C without aeration. 1 mL of preculture was diluted in 49 mL MRS supplemented with 0.4 M – 0.7 M NaCl and incubated for 22 hours. Cells were washed three times with ice-cold ddH<sub>2</sub>O and resuspended in 900 μL ddH<sub>2</sub>O. 50 μL aliquots were electroporated with 2.5 kV and 200 Ω (25 μF).

### ***3.2.3. L. reuteri CD037***

#### ***Cultivation experiments***

*L. reuteri* CD037 was incubated overnight in light MRS, dark MRS and M17 broth. Growth and cell aggregation in light MRS were protocolled after adding D-glucose, D-sucrose, D-maltose, D-xylose, D-galactose, D-fructose and Ca-L-lactate (10 g/L).

Agglutination was also compared in light MRS supplemented with different  $Mg^{2+}$  (0, 0.001, 0.02, 0.05 M) or  $Ca^{2+}$  (0, 0.001, 0.02, 0.05 M) concentrations using  $MgSO_4$  and  $CaCl_2$ .

### ***Optimization of electrical parameters and initial protocol for electroporation***

Based on Axelsson and Ahrne (1990), an overnight culture of *L. reuteri* CD037 was grown in MRS at 37 °C and diluted in 40 mL fresh MRS to an  $OD_{600}$  of 0.2. After growth at 37 °C to an  $OD_{600}$  of 0.5 – 0.7, cells were harvested by centrifugation at 4 °C, washed three times in ice-cold ddH<sub>2</sub>O and once in 30 % PEG<sub>1500</sub>. The cells were suspended in 30 % PEG<sub>1500</sub> leading to an  $OD_{600}$  of 10 to 20 and aliquoted into 40 µL portions. The suspensions were electroporated without addition of plasmids to determine survival rates. Different combinations of voltage and resistance were applied; capacity was set at 25 µF.

Table 5. Different combinations of voltage and resistance at 25 µF.

<b>V</b>	1,000	1,000	1,500	1,500	2,000	2,000	2,500	2,500
<b>Ω</b>	800	400	800	400	400	200	400	200

The electrotransformation protocol was repeated, adding 1 µg of pCD plasmids, respectively, to the aliquoted cells. DNA and cells were mixed and held 10 min on ice prior to electroporation. After application of the electric pulse, cells were diluted in 360 µL MRS and incubated for 2 hours at 37 °C, before plating them on MRS agar containing 5 µg/mL chloramphenicol.

### ***Protocol modifications***

The initial protocol was conducted with minor changes. Glycine was added to the growth medium as a cell weakening agent. An overnight culture was diluted in MRS containing 2 % glycine. Washing and electroporation were performed as described above. After application of the pulse, cells were incubated for 4 hours at 37 °C in MRS supplemented with 0.5 M sucrose as an osmotic stabilizer.

### **Washing solutions**

Further actions were taken concerning the composition of the washing solution.

*L. reuteri* CD037 was grown in MRS overnight, incubated in MRS + 2% glycine and harvested. Following centrifugation, cells were resuspended in either 3 mL high salt buffer (0.75 M NaCl, 0.05 M Tris, 0.025 M EDTA), 0.5 M NaCl, 0.05 M NaOH or 0.025 M NaOH. Cells were incubated for 15 to 20 min on ice and then washed three times with ddH<sub>2</sub>O and once with PEG<sub>1500</sub> 30 %. The aliquots were incubated with pCDWV015 10 min on ice before applying a single pulse of 2000 V/200 Ω (25 μF). Post-pulse the cells were diluted in MRS 0.5 M sucrose and incubated 4 hours at 37°C. Plating on MRS agar was performed to confirm cell survival.

A succeeding experiment was conducted by growing *L. reuteri* CD037 in MRS to an OD<sub>600</sub> of ~ 0.6. The culture was harvested by centrifugation and incubated for either 15 minutes in 50 mM EDTA on ice, or for 20 minutes in lysozyme (25 mg/mL) at 37 °C. The cell pellet was washed three times in ddH<sub>2</sub>O. The resulting cell solutions were examined under the microscope.

### **Glycine concentrations**

An overnight culture was diluted in MRS containing different concentrations of glycine (0, 1, 2 or 3 %). After reaching a sufficient density (OD<sub>600</sub> ~ 0.6), each culture was split in 4 fractions and incubated 15 min on ice with 0, 1, 10 or 50 mM MgCl<sub>2</sub>, respectively. MgCl<sub>2</sub> was further used as washing solution, following one washing step with 30 % PEG<sub>1500</sub>. Due to the increased ionic content of the cell suspension, 2000 V/400 Ω/25 μF were applied to the aliquots incubated with 1 μg of pCDWV01.

### **Lithium-acetate method**

The method was applied as described in 3.2.2. after supplementing light MRS either with xylose, glucose or galactose.

### **Protocol for *Lactococcus lactis***

(Holo and Nes, 1989)

The original protocol is based on *Lactococcus lactis*. Growth conditions were adapted from 30 °C in M17 medium with 0.5 % glucose to 37 °C in MRS with 0.5 % glycine. *L. reuteri* CD037 was grown until  $OD_{600} = 0.5 - 0.8$  was reached, diluted 1:100 in MRS containing 0.5 % glucose, 0.5 M sucrose and glycine (0, 2, 3, 4, 6 or 8%) and further incubated to  $OD_{600} = 0.2 - 0.7$ . The culture was washed twice in ice-cold 0.5 M sucrose, once in 10 % glycerol and resuspended in 0.5 mL glycerol. Aliquots of 40  $\mu$ L were electroporated at 2 kV and 200  $\Omega$  (25  $\mu$ F) and suspended in 960  $\mu$ L ice-cold MRS supplemented with 0.5 % glucose, 0.5 M sucrose, 20 mM  $MgCl_2$  and 2mM  $CaCl_2$ . Electroporated cells were held on ice for 5 minutes and then incubated for 2 hours at 30 °C prior to plating on MRS chloramphenicol.

### **Improved protocol**

(Thompson and Collins, 1996)

The protocol was initially used for *L. plantarum*, hence we adapted it for *L. reuteri* CD037. Cells were grown over night at 37 °C in light MRS containing 1% glucose. The cell culture was diluted to an  $OD_{600}$  of 0.2 in 50 mL MRS with 0.5 % glucose, 0.3 M sucrose and 8 % glycine. After 4.5 hours of incubation at 37 °C, cells were harvested, washed twice in ddH<sub>2</sub>O, held 5 minutes on ice in 50 mM EDTA, washed twice with ddH<sub>2</sub>O again, once with 0.3 M sucrose and then resuspended in 0.5 mL 0.3 M sucrose. Cells were diluted after electroporation (2.5 kV, 200  $\Omega$ , 25  $\mu$ F) in 360  $\mu$ L pre-warmed MRS supplemented with 0.5 M sucrose and incubated for 2 hours at 37 °C. The suspension was plated on MRS agar containing chloramphenicol.

## **3.2.4. Biomolecular methods**

### **Preparation of plasmid DNA**

Plasmids (pCD) were prepared from *E. coli* JM109 using either the midi-prep kit (Promega, Germany) or the mini-prep Nucleospin plasmid kit (Macherey-Nagel, Germany). *E. coli* JM109 containing pCDWV01 was grown in LB supplemented with chloramphenicol, while ampicillin was used for selection of *E. coli* JM109 carrying the other pCD plasmids.

## Polymerase Chain Reaction

PCRs were performed to confirm transformation of lactobacilli with pCD plasmids.

### KOD

The KOD polymerase has a 3' → 5' exonuclease-dependant proofreading function and was purchased from Merck (Germany).

### KOD protocol

The master mix was usually split to 50 µL aliquots.

Table 6. Composition of KOD mastermix.

KOD buffer 10x	5.0 µL
dNTPs (2 mM each)	5.0 µL
MgCl <sub>2</sub> (25 mM)	3.0 µL
KOD DNA polymerase (2.5 U/µL)	0.4 µL
sense primer (10 pmol/ µL)	2.0 µL
anti-sense primer (10 pmol/ µL)	2.0 µL
template DNA (0.006 – 6 ng)	1.0 µL
ddH <sub>2</sub> O	added to 50 µL

Table 7. Thermocycler program for KOD polymerase.

Step 1	98 °C	35 sec
Step 2	T <sub>m</sub> – 5 °C	15 sec
Step 3	72 °C	25 sec/2 kbp
Step 4	72 °C	5 min

Steps 1 to 3 were repeated 30 times.

### Hybrid Pol

(Novagen, USA)

Table 8. Composition of HybridPol mastermix.

Hybrid Pol buffer 10x	5 µL
dNTPs (10 mM)	0.5 µL

sense primer (10 pmol/ $\mu$ L)	0.75 $\mu$ L
anti-sense primer (10 pmol/ $\mu$ L)	0.75 $\mu$ L
RNase A	0.1 $\mu$ L
DMSO	0.5 $\mu$ L
MgCl <sub>2</sub> (50 mM)	2 $\mu$ L
Hybrid polymerase	0.5 $\mu$ L
ddH <sub>2</sub> O	add to 50 $\mu$ L

Table 9. Thermocycler program for Hybrid polymerase.

Step 1	94 °C	3 min
Step 2	94 °C	15 sec
Step 3	Tm -5 °C	30 sec
Step 4	72 °C	1 min
Step 5	72 °C	10 min

Steps 2 to 4 were repeated 35 times.

### *Gel electrophoresis*

Agarose gel electrophoresis was performed at 130 V for analytical gels (15 – 20 minutes) and with 110 V for preparative gels (30 – 40 minutes). Samples were mixed with 6x BX buffer (0.25 % bromphenol blue, 0.25 % xylene cyanol, 30 % glycerol). 6  $\mu$ L of 2-log DNA ladder (0.1 – 10 kb; New England Biolabs, UK) were applied to a 1 % agarose gel (1% agarose in 1xTAE buffer) to determine DNA length of fragments. Gels were supplemented with ethidiumbromide ( $9 \times 10^{-7}$  M) and run in TAE buffer (40 mM Tris-acetate, 100 mM EDTA).

### *DNA extraction from gels and purification*

We used two distinct kits for extraction and purification of PCR products, either the QIAEX II Gel Extraction Kit (QIAGEN, USA) or the PCR clean up kit (Macherey-Nagel, Germany).

## 4. Results and Discussion

### 4.1. Transformation of *L. reuteri* CD037

#### 4.1.1. Growth media

##### *Selection*

Our first aim was to identify an optimal growth medium for *L. reuteri* CD037 and then to alter it in favour of cell wall weakening processes. Several media have already been used in successful electroporation protocols dealing with the type strain *L. reuteri* DSM 20016, like MRS bouillon (Lizier *et al.*, 2010) and LCM medium (Axelsson and Ahrne, 1990). It is advantageous to use highly cell growth-promoting media, as effector agents like glycine, sucrose and salts are known to decelerate growth (Holo and Nes, 1989; Palomino *et al.*, 2010).

Although we had guidelines and electroporation models from articles on the other *L. reuteri* strains, *L. reuteri* CD037 turned out to be the most challenging strain of all lactic acid bacteria we were examining. The most obvious feature was its clumping in liquid MRS bouillon, which was not observed in the type strain. This property aggravated accurate OD<sub>600</sub> measurements. Furthermore we thought that exopolysaccharides responsible for clumping might obstruct absorption of plasmids to competent cells and consequently inhibit transformation.

Therefore we compared three different frequently used LAB media not only for their ability to propagate *L. reuteri* CD037 but also to enhance cell dispersion.

Our strain showed very scarce growth in commercial MRS Bouillon and GM17 while clumping was still obvious. Only light MRS seemed to match the nutritional needs of *L. reuteri* CD037, although unable to inhibit cell aggregation. We decided to use variations of light MRS for our further examinations.

##### *Carbon source*

Microorganisms are usually able to catabolize a wide variety of carbons and to even prosper in poor conditions depending on the metabolic pathways they possess. In contrast, most LAB show a limited ability to synthesize essential metabolites like amino acids and nucleic acids probably due to their specialization on nutrient rich



habitats. However, as they are surrounded by an excess of different sugars, they might be able to catabolize those sugars in different metabolic pathways.

*Lactococcus lactis* was shown to produce less exopolysaccharides (EPS) when grown on fructose rather than on glucose (Petronella *et al.*, 1999). This was a result of the low activity of fructosebisphosphatase that is involved in production of sugar precursors when cells are grown on fructose and of the fact that the EPS precursors are used both in EPS biosynthesis and in cell wall formation.

Experiments indicate that precursors, when scarce, are preferentially used for the formation of cell wall sugars. A lack in precursors will therefore result in less EPS production. This phenomenon is common to many lactobacilli, as shown in *L. casei*, *L. rhamnosus*, and *L. delbrueckii* (Cerning *et al.*, 1994; Gamar *et al.*, 1997; Grobber *et al.*, 1997) cultivated in fructose. Hence, we tested a putative impact of different carbon sources on agglutinative growth of *L. reuteri* CD037.

The choice of carbon source did influence the shape of growth curves (Fig.3), indicating that different metabolic pathways were active and in some cases had to be induced. Nevertheless, there was no effect on cell clumping. Agglutination occurred with glucose, saccharose and maltose equally. A following experiment using galactose, lactate, fructose and xylose led to the same results. We also tested reduced concentrations of glucose to directly limit production of EPS precursors which resulted in lower growth but unchanged agglutination.

As glucose is typically used as a carbon source in microbiology and did not display any disadvantages compared to the other tested carbon sources, we decided to use it further on in MRS bouillon according to the manufacturers recommended concentration.

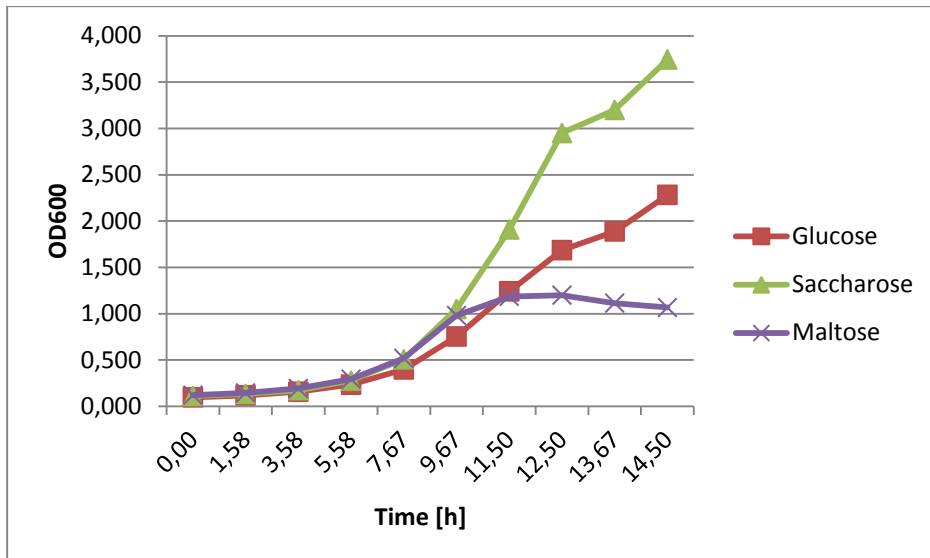


Figure 3. Growth curves of *Lactobacillus reuteri* CD037. Cells were grown at 30 °C in MRS Bouillon containing different carbon sources.

### Divalent cations

Agglutination might also be a stress response, due to a lack of specific nutrients like vitamins or trace elements. In fact, certain micronutrients like ions might have an effect in either a metabolic or physicochemical way. Minerals like magnesium, calcium and iron are essential in metabolism and might therefore have a critical impact on growth performance of microorganisms.

Considerable studies state that divalent cations like magnesium and calcium are able to induce morphological changes in microorganisms. Many sites on gram-positive walls harbouring carboxyl, phosphoryl or secondary polymers have the potential to bind divalent cations (Beveridge, 1989). Yousef and Espinosa-Urgel (2007) characterized proteins involved in cell-cell-adhesion and cell surface interactions and found that many of these proteins contained  $\text{Ca}^{2+}$ -binding domains, a further mechanistic explanation for a putative effect of  $\text{Ca}^{2+}$  ions.

To this end, we decided to test different magnesium and calcium concentrations in the growth medium. The addition or reduction of those cations resulted in no obvious effect on *L. reuteri* CD037. All samples agglutinated heavily with long filament chains being visible under the microscope. Freely dispersed filaments were scarce.

Up to date findings on the effect of divalent cations in the growth medium do not seem to be coherent. Cell aggregation was shown to be enhanced by calcium ions for example in *E. coli* (Onoda *et al.*, 2000), *C. trachomatis* (Majeed *et al.*, 1993) and *S. hygroscopicus* (Dobson L. F. and O'Shea D., 2008). In contrast, magnesium increased surface hydrophobicity of *E. coli* (Latrache *et al.*, 2000), while Dobson *et al.* (2008) found *S. hygroscopicus* to be hydrophilic and freely dispersed when grown in magnesium-rich medium. Cell surface hydrophobicity was usually determined by measuring the contact angle between a bacterial cell layer and a deposited drop of distilled water (Thaveesri *et al.*, 1995).

However, we did not observe any effect of magnesium or calcium concentrations on agglutination of *L. reuteri* CD037.

#### 4.1.2. Washing solutions

Preparation of electrocompetent cells always includes washing steps to remove salts from the cell solutions and hence increase the resistance of the sample. High resistance may also be sustained by the addition of glycerol or sucrose (Eynard and Teissié, 2000). Additionally washing solutions can have a beneficial effect on cell dispersion. Indeed, ions and chelators are frequently used as additives in initial washing steps to interfere physicochemically with extracellular polysaccharides or cell membranes. Once again there is no golden rule how and which agents to apply. For example, Berthier *et al.* (1996) reported a decrease in transformation efficiency of *L. sake* when  $MgCl_2$  was added to the electroporation buffer, while in contrast washing the same strain in  $MgCl_2$  increased the efficiency. The effect of washing can also be as simple as to remove aggregation-promoting factors from the supernatant, as has been described by Jankovic *et al.* (2003) for aggregative *Lactobacillus gasseri*.

Consequently, we tested different washing solutions for their ability to possibly counteract agglutination of *L. reuteri* CD037. Washing steps with 0.5 M NaCl, 0.05 M NaOH or 0.025 M NaOH remained ineffective in comparison to distilled water usage. Also incubation with high salt buffer (0.75 M NaCl, 0.05 M Tris, 0.025 M EDTA), EDTA (50 mM) or even mild lysozyme treatment (25 mg/mL) was not able to induce cell separation.

The finding that neither carbon source and cations in the growth medium nor the variation of washing solutions promoted cell dispersion made us doubt that an extracellular matrix has to be blamed for the aggregative growth of *L. reuteri* CD037. The filaments might also be formed by a defect in autolytic enzymes needed for complete cell division, in which case little can be done to increase cell dispersion while keeping cells viable. In fact, Williamson and Ward published in 1981 an article on autolysin deficiency in *B. subtilis* and *S. pneumonia*. They described the morphology of mutants as chains of non-separated bacilli which applies perfectly to *L. reuteri* CD037. Additionally they observed a decreased porosity of the cell wall as a direct result of autolysin deficiency. A change in cell wall permeability would have serious impacts on transformability of strains and explain the difficulties faced with *L. reuteri* CD037.

#### 4.1.3. Alterations of the cell wall

##### *Glycine*

As described before numerous substances like lysozyme, penicillin, and threonine are used regularly in electroporation studies to induce changes in the cell walls of reluctant gram-positive bacteria. A regularly recurring cell wall weakening compound in *Lactobacillus* transformation is glycine (Thompson and Collins, 1996; Berthier *et al.*, 1996; Holo and Nes, 1989).

We journalized growth of *L. reuteri* CD037 at different temperatures and glycine concentrations (Fig. 4 and 5) to evaluate suitability of glycine for our purposes. The concentrations of both 1% and 2% glycine led to a growth reduction of more than 50% compared to glycine-free medium. The growth inhibition at 4% glycine was already too unfavourable for a fast and easy-to-apply approach.

It was also obvious that incubation at 37 °C supported faster growth by means of needing less time to reach the log-phase. As a result we decided to incubate *L. reuteri* CD037 at 37 °C in all succeeding experiments.

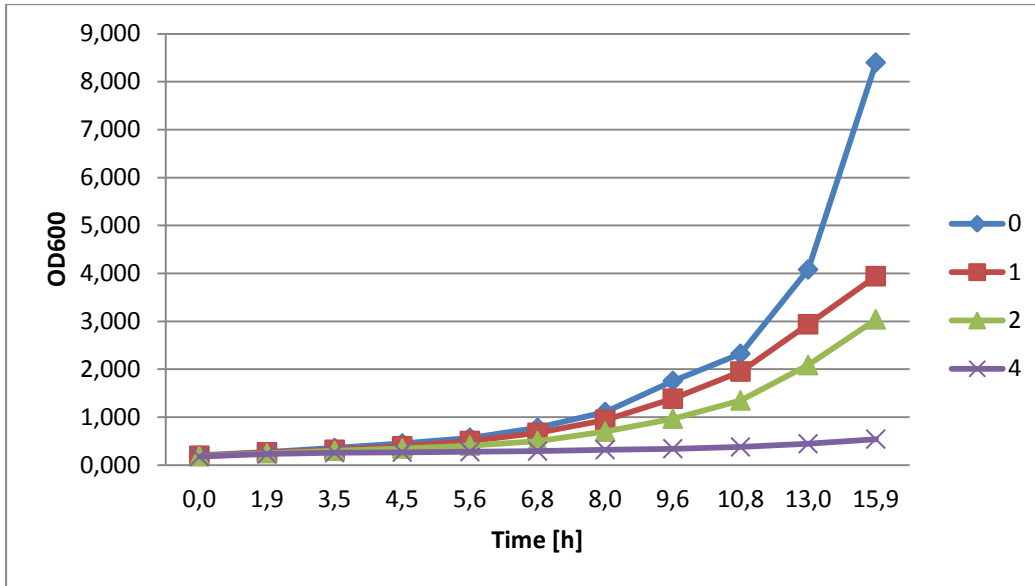


Figure 4. Growth curve of *L. reuteri* CD037 at different glycine concentrations ([%]). Bacteria were grown in MRS Bouillon with 1% glucose at 30 °C.

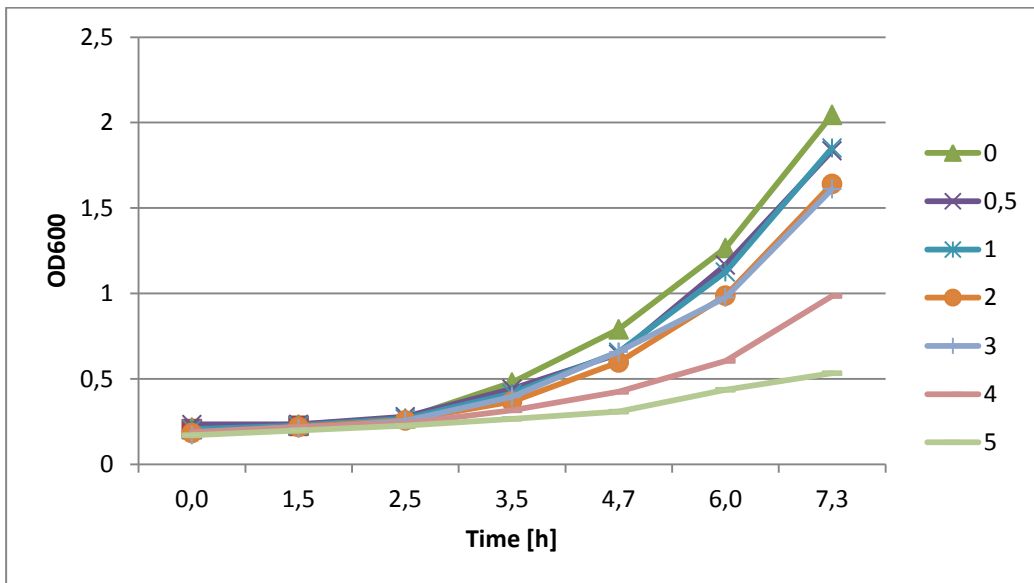


Figure 5. Growth curve of *L. reuteri* CD037 at different glycine concentrations [%]. Cells were grown in MRS Bouillon with 1% glucose at 37 °C and different glycine concentrations.

### Glycine and sucrose

Holo and Nes (1989) investigated the effect of a combination of sucrose and glycine in growth medium on electrotransformation efficiencies in *L. lactis*. They reported an

increase in transformability when sucrose was added. The osmotically stabilizing agent led to growth retardation when it came to speed and at the same time allowed for growth in higher glycine concentrations. Other stabilizers, like rhamnose, mannitol, sorbitol or maltose, are known but not as extensively tested in electroporation as sucrose. We therefore decided to assess the impact of 0.3 M sucrose in MRS medium on growth of *L. reuteri* CD037.

The application of the protocol led to similar results as predicted by the authors. Pronounced growth was still visible at 4% glycine in contrast to unstabilized medium bearing the same glycine concentration (Fig.6). In contrast to the original article, we did not observe a delay in log-phase occurrence and we were also not able to sustain growth in 6% glycine or higher.

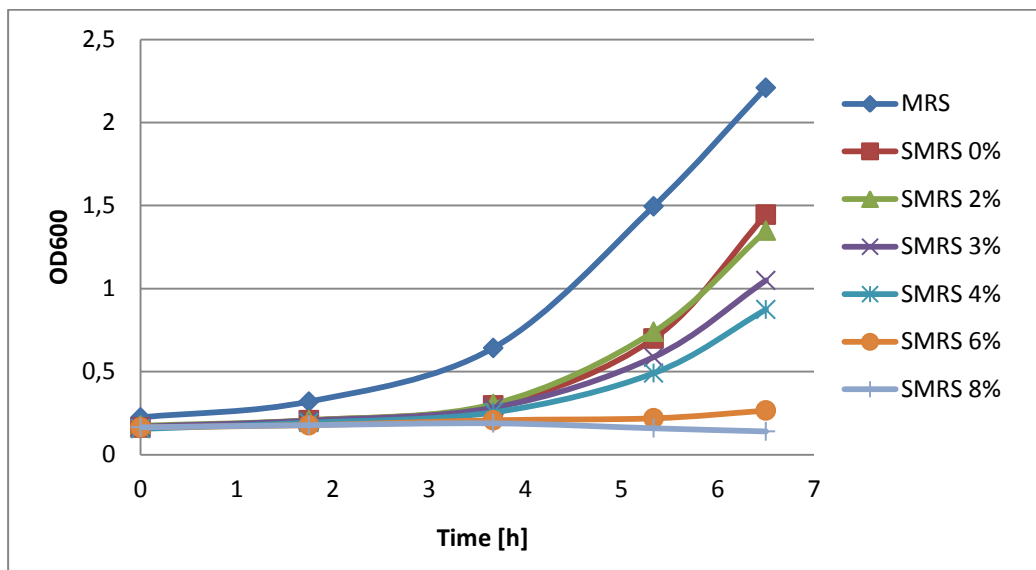


Figure 6. *L. reuteri* CD037 grown at different glycine concentrations [%] supplemented with sucrose. Cells were grown at 37 °C in plain MRS bouillon containing 1% glucose and MRS with 1% glucose plus 0.3 M sucrose (SMRS) at different glycine concentrations (%).

#### 4.1.4. Optimization of electrical parameters

Adjusting the voltage and the electropulsators resistance has critical impacts on transformation efficiency.

To determine which electrical settings are suitable for *L. reuteri* CD037, we applied Axelsson and Ahrnes (1990) protocol with minor modifications (3.2.3.). Aliquots were diluted to an  $OD_{600} = 11-19$ , resulting in a total count of about  $10^8$  CFU/40  $\mu$ L aliquot. We determined survival rates after 4 washes and electroporation under various voltage and resistance combinations (Tab. 10). The resulting disparity in survival depending on the different settings was only moderate. Thus we agreed to use 2000 V/400  $\Omega$ /25  $\mu$ F and 2500 V/400  $\Omega$ /25  $\mu$ F for further experiments as they displayed the lowest survival rates and should therefore have the highest impact on cell structure.

Table 10. Cell survival at different voltage and resistance combinations. Washed *L. reuteri* CD037 were treated with different combinations of voltages and resistances with a capacity of 25  $\mu$ F and plated on MRS agar (surviving cells/aliquot containing  $\sim 10^8$  CFU).

V	$\Omega$	surviving cells
1000	800	$4 \cdot 10^6$
1000	400	$5 \cdot 10^6$
1500	800	$4 \cdot 10^6$
1500	400	$8 \cdot 10^6$
2000	400	$1 \cdot 10^6$
2000	200	$2 \cdot 10^6$
2500	400	$7 \cdot 10^5$
2500	200	$3 \cdot 10^6$

#### 4.1.5. Electroporation protocols

##### *Initial protocol*

Electrotransformation of gram-positive bacteria usually requires at least one step where the cell wall is weakened. Axelsson and Ahrne published in 1990 a simple but nevertheless effective method to electroporate the *L. reuteri* type strain DSM 20016.

The protocol included growth in Lactobacillus Carrying Medium supplemented with glucose until mid-log phase was reached ( $OD_{600} = 0.5 - 1$ ), followed by three washes in distilled water. Cells were electroporated in 30% PEG<sub>1500</sub> and incubated post-pulse 1.5 hours in growth medium. Axelsson and Ahrne reported transformation efficiencies ranging from  $10^7$  to  $10^8$  transformants/ $\mu\text{g}$  DNA.

We repeated a slightly altered electrotransformation protocol as described previously (3.2.3.) for determination of electrical parameters and added 1  $\mu\text{g}$  of pCD plasmids, respectively, to the aliquoted cells. Post-pulse incubation time was set at 2 hours, which is assessed to give cells enough time to recover and resume bacterial protein synthesis as the CAT gene itself is known to be rapidly expressed.

Neither the exposition to 2000 V/400  $\Omega$ /25  $\mu\text{F}$  nor to 2500 V/400  $\Omega$ /25  $\mu\text{F}$  resulted in transformants.

As a consequence, we modified the initial protocol by diluting the overnight culture in MRS containing 2 % glycine. After we applied the pulse, we incubated cells 4 hours at 37 °C in MRS supplemented with 0.5 M sucrose as an osmotic stabilizer. We obtained no transformed cells.

As we suspected the exopolysaccharides to possibly hinder successful transformation, we further adjusted the protocol. We grew cells in MRS overnight, incubated them in MRS supplemented with 2% glycine and harvested cells as usual. Following centrifugation we resuspended cells in either NaCl (0.5 M) or NaOH (0.05 M). Plating on MRS agar confirmed sufficient survival of cells; still we obtained no transformants on chloramphenicol supplemented agar.

Our next step included alterations of the washing solution. We decided to use  $\text{MgCl}_2$  instead of pure  $\text{dH}_2\text{O}$ . *L. reuteri* CD037 was repeatedly reluctant to this treatment as seen with NaCl and NaOH before.

### ***Lithium-acetate protocol***

Lithium acetate is an agent widely used in yeast electroporation yielding high numbers of transformants (Wu and Letchworth, 2004; Cregg and Russell, 1998; Thomson *et al.*, 1998). It is used for several transformation methods of intact yeast cells followed by a heat shock (Ito *et al.*, 1983) or in combination with dithiothreitol for electroporation (Thompson *et al.*, 1998).

In 2007, Papagianni *et al.* applied a pre-treatment to *L. lactis* strains using lithium acetate and DTT before conduction of electroporation. According to the



authors the combination of lithium acetate with a reducing agent is able to modify porosity of treated cell walls and hence increase their permeability.

We incubated harvested *L. reuteri* CD037 in lithium acetate supplemented with DTT and washed thoroughly with EDTA and sucrose. The treatment did not result in transformants. We supplemented the growth medium either with xylose, glucose or galactose prior to lithium acetate pre-treatment, but did not detect any differences in growth, clumping or transformation efficiency.

### **High salt protocol**

In 2010, Palomino *et al.* introduced a cost-efficient method to electrotransform lactobacilli. They grew pre-cultures in 0.9 M NaCl and conducted washing of cells with ultra-pure water before electroporation. This simple procedure was able to transform certain strains of *L. casei*, *L. delbrueckii*, *L. paracasei*, *L. plantarum* and *L. acidophilus*.

The effect was speculated to be due to reduced peptidoglycan crosslinking which is an advantageous prerequisite for electroporation (Palomino *et al.*, 2010). Salts are known to act chaotropic, disrupting hydrogen bonds and van der Waals bonds, which may result in sufficient cell wall weakening in certain strains. Osmotic stress induced by growth in high salt concentrations has also been reported to increase intrinsic autolytic activity and sensitivity to lysis in *L. delbrueckii* and *L. casei* (Koch *et al.*, 2007; Piuri *et al.*, 2005).

A work on *Bacillus subtilis* (Thwaites *et al.*, 1990) confirmed the effect of high salt concentrations in rendering cell walls more ductile, while changes in pH and even lysozyme treatment had little influence on mechanical properties. Ions are presumably able to change the polymer conformation of peptidoglycan and to diminish the electrostatic forces between charged groups in the cell wall.

*L. reuteri* CD037 was too osmosensitive to grow in 0.9 M NaCl, hence we adjusted the salt concentrations to 0.4, 0.6 and 0.7 M and proceeded with the protocol as recommended (3.2.2.). We were not able to produce transformants with the conditions proposed by Palomino.

### **Improved protocol**

(Thompson and Collins, 1996)

In 2010, Lizier *et al.* transformed *L. reuteri* DSM 20016 and *L. reuteri* strains isolated from chicken crops to compare the effectiveness and activity of different expression vectors. The protocol for their experimental setup was derived from Thompson and Collins (1996) who further improved the effect of glycine by adding sucrose. Although sucrose itself is inhibitory towards growth it increases transformation efficiencies when combined with glycine. Sucrose allowed for high glycine concentrations as up to 8 %, probably by acting as an osmotic stabilizer and preventing lysis of weakened cells.

We followed the described protocol using sucrose and 6% glycine. Survival rate after electroporation was  $2 \cdot 10^5$  cfu/40  $\mu$ L (~ 0.2 % survival), but we did not obtain transformants.

## 4.2. Comparison of different electroporation protocols

### 4.2.1. Initial protocol

The initial protocol was applied as described before including growth in MRS supplemented with the cell wall weakening compound glycine. The treatment revealed no effect on *L. buchneri* CD034, and *L. buchneri* CD035 while *L. plantarum* CD032 and *L. plantarum* CD033 were both successfully transformed (Tab.11). Lowering the field strength of 2.5 kV to 2 kV in electroporation of *L. plantarum* CD032 increased the number of transformants. This could be partly due to higher cell survival, but is not a sufficient explanation as electroporation efficiency differences for pCDE194 were minimal while efficiency for pCD256 rose 1000-fold compared to 2.5 kV.

Transformation efficiencies are also highly dependent on the plasmids used. For instance, pCDWV02 was not able to establish in the *L. plantarum* strains CD032 and CD033.

Table 11. Transformation efficiencies of *L. plantarum* (CD032 and CD033), *L. buchneri* (CD034 and CD035), and *L. reuteri* (CD037) strains grown in 1 % glycine. n.t. not tested; cells were aliquoted to an OD<sub>600</sub> of 11-19. survival = CFU/aliquot after electroporation, plated on MRS agar.

Strain	Survival	Transformants/ $\mu\text{g}$ DNA			
		pCDWV02	pCD256	pCDE194	pCDWV01
CD032 (2 kV)	n.t.	-	$6 * 10^3$	$1 * 10^2$	$3 * 10^3$
CD032 (2.5 kV)	n.t.	-	-	$7 * 10^2$	$6 * 10^1$
CD033	n.t.	-	$2 * 10^3$	$4 * 10^2$	$1 * 10^4$
CD034	n.t.	-	-	-	-
CD035	n.t.	-	-	-	-
CD037	$7 * 10^5$	-	-	-	-

#### 4.2.2. Lactococcus high efficiency protocol

The protocol described by Holo and Nes (1989) is highly similar to the initial protocol. It is distinguished by

- addition of sucrose to the growth medium which improves cell stability and allows for higher glycine concentrations
- glycerol containing washing solutions to avoid arcing of the sample, and
- divalent cations in the recovery medium to additionally stabilize cell membranes (Munreet *et al.*, 2007).

Holo and Nes achieved efficiencies of  $10^3$  up to  $10^7$  transformants per  $\mu\text{g}$  DNA depending on the *L. lactis* strain used.

The protocol proved successful for electrotransformation of *L. lactis* MG1363, even though with moderate efficiencies (Tab. 12). It was remarkable that colonies of two distinct sizes were obtained. Both types were able to proliferate in liquid medium containing chloramphenicol which indicates that plasmid was uptaken by both colony types and disprove a false positive result.

Table 12. Transformation efficiency of *L. lactis* MG1363 following the lactococcus high efficiency protocol. Colonies differed in size and were counted separately (small colonies + large colonies). survival = CFU/aliquot after electroporation plated on MRS agar.

Strain	Survival	Transformants/ $\mu\text{g}$ DNA			
		pCDWV02	pCD256	pCDE194	pCDWV01
MG1363	$2 * 10^8$	$7 * 10^0 + 3 * 10^1$	$- + 5 * 10^2$	-	$2 * 10^2 + 4 * 10^4$

### 4.2.3. Lithium acetate method

We prepared our working strains according to the lithium acetate method prior to electroporation. *L. plantarum* CD032 was impaired strongly in survival and as a consequence did not result in transformants, while *L. plantarum* CD033 and *E. faecium* CD036 were more robust – nevertheless yielding low transformation efficiencies (Tab.13). Moderate efficiencies were obtained with *L. buchneri* CD034 and partly with *L. lactis* MG1363.

Both, *E. faecium* CD036 and *L. lactis* MG1363 were equally transformable after storage at -80 °C with no reported losses of viability.

Table 13. Transformation efficiencies of *L. plantarum* CD032 and CD033, *L. buchneri* CD034, *E. Faecium* CD036, *L. reuteri* CD037, and *L. lactis* MG1363 strains subjected to lithium acetate and DTT prior to electroporation. Small and large colonies were observed in some cases (small colonies + large colonies). survival = CFU/aliquot after electroporation plated on MRS agar.

Strain	Survival	Transformants/ $\mu$ g DNA			
		pCDWV02	pCD256	pCDE194	pCDWV015
CD032	$5 * 10^2$	-	-	-	-
CD033	$2 * 10^8$	-	$3 * 10^1$	$4 * 10^0$	$7 * 10^1$
CD034	$2 * 10^8$	$1 * 10^2$	$2 * 10^3$	$2 * 10^3$	$2 * 10^3$
CD036	$4 * 10^8$	$3 * 10^1 + 2 * 10^1$	-	-	$6 * 10^1$
CD037	$2 * 10^5$	-	-	-	-
MG1363	$8 * 10^7$	-	-	-	$2 * 10^1 + 3 * 10^3$

### 4.2.4. High salt protocol

The promising high salt protocol failed to transform most of our strains, although proved to be very efficient in several lactobacillal species (Palomino *et al.*, 2010).

In addition to testing the protocol, we also determined the effect of two varying electrical settings for *L. plantarum* CD032. Lower applied voltage resulted in higher survival rates, but no transformants, while increased voltage favoured uptake of pCDE194, albeit in low numbers (Tab.14).

Our strains showed different degrees of osmosensitivity. While *L. buchneri* CD034 was able to grow in 0.9 M NaCl, we had to reduce the concentration to 0.7 M for *L. plantarum* CD032 and CD033. The protocol was not suitable for *L. lactis* MG1363, as this strain was even too sensitive to grow in 0.7 M NaCl.

*L. buchneri* CD034 was the only strain transformable by the method, displaying solid efficiencies.

Table 14. Transformation efficiencies of *L. plantarum* CD032 and CD033, *L. buchneri* CD034, *L. reuteri* CD037, and *L. lactis* MG1363 grown in high salt concentrations prior to electroporation (200  $\Omega$ /25  $\mu$ F). n.t. not tested. survival = CFU/aliquot after electroporation plated on MRS agar.

Strain	Survival	Transformants/ $\mu$ g DNA			
		pCDWV02	pCD256	pCDE194	pCDWV01
CD032 (2 kV; 0.7 M NaCl)	$1 * 10^8$	-	-	-	-
CD032 (2.5 kV; 0.7 M NaCl)	$4 * 10^7$	-	-	$1 * 10^1$	-
CD033 (0.7 M NaCl)	n.t.	-	-	-	-
CD034 (0.9 M NaCl)	$1 * 10^8$	$1 * 10^2$	$2 * 10^3$	$2 * 10^3$	$5 * 10^3$
CD037 (0.4 M NaCl)	$7 * 10^6$	-	-	-	-
CD037 (0.6 M NaCl)	$3 * 10^6$	-	-	-	-
MG1363 (0.7 M NaCl)	-	-	-	-	-

## 5. Conclusion

Even if assigned to the same species, strains show huge variation in transformability and optimal growth conditions. A protocol working for one strain might not be suitable for the other one.

According to Rossello-Mora *et al.* (2001), a species is defined as „a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property“. Nevertheless many characteristics are not shared. It is known for example for probiotics that all health benefits and effects are highly strain-specific and not species-specific (Madsen, 2006).

In addition to the wide definition of species, the group of lactic acid bacteria itself is characterized by a huge heterogeneity, which spreads as far as to strain level. Even within our small setting of experiments comparing two strains of *Lactobacillus plantarum* and *Lactobacillus buchneri* each, the differences were astonishingly obvious. Strains differed in as simple characteristics as growth dramatically. While *L. buchneri* CD034 and the *L. plantarum* strains showed steady growth in our experiments, both *L. buchneri* CD035 and *L. reuteri* CD037 were extremely sensitive. Colonies picked from refrigerated agar plates did not recover in fresh liquid medium, which was also true for colonies grown longer than two days and taken directly from the incubator.

On the other hand once the working strains were rendered electrocompetent they were in general robust in means of storage and transformability. Electrocompetent cells frozen in PEG<sub>1500</sub> could be pulsed after thawing without a significant decrease in transformation efficiency.

In accordance to literature, the lithium acetate method affirmed three noteworthy phenomena observed before with the initial and high efficiency protocol:

- Two strains of the same species will not imperatively react in the same way to the same treatment. This is at first sight obvious for the survival rates and transformation efficiencies of *L. plantarum* CD032 and CD033 as well as for the *L. buchneri* strains CD034 and CD035.

- Even differences within a clonal cell suspension are possible as seen for *L. reuteri* CD037 with pCDWV02 and *L. lactis* MG1363 with pCDWV01. Both displayed large and small colonies. The same effect was detected in *L. lactis* MG1363 using the lactococcus high-efficiency protocol.
- The choice of plasmid is crucial. pCDWV01 turned out to be the most effective plasmid. This specific plasmid was uniquely characterized by the absence of an *E. coli* origin which might compromise efficient replication in certain lactobacilli.

Independent of electroporation protocol or plasmid choice *L. reuteri* CD037 remained reluctant to electroporation. Also the cause and putative reason for agglutinative growth of *L. reuteri* CD037 rests elusive. A mechanism favouring growth of biofilms and rendering the strain more robust in the gastrointestinal tract would be possible. More probably the strain is subject to a mutation affecting the autolytic process and hence complete cell division. As mentioned before such an autolytic deficiency would heavily impair permeability of the cell wall and require more aggressive treatments. We did not observe such pronounced agglutination in any other working strain.

The list of possible obstacles to electroporation is manifold, ranging from exopolysaccharides and agglutination to more uncommon reasons. For instance, Berthier *et al.* (1996) identified 30 % PEG as a complete inhibitor of electrotransformation of *L. sake* cells which was utilized by default in our experiments. PEG is widely used as electroporation or storage solution and was also shown to increase electroporation of *L. plantarum* and a different *L. sake* strain (Bringel and Hubert, 1990; Aukrust and Blom, 1992). Aukrust *et al.* (1993) described loss of electrocompetence of some strains when kept too long on ice before electroporation.

Resident plasmids in the industrial wild-type *L. reuteri* CD037 also could possibly interfere with transforming plasmids and hinder establishment of transformants. Transforming plasmids are not equally suitable for all strains depending on their size, DNA restriction, modification, and replication mechanism.

The impact of a huge number of parameters like growth phase, final concentration of cells, the choice of plasmids, composition of electroporation solution, washing solution, growth media, electroporation conditions like field strength, pulse

duration, number of pulses, resistance, and many more aggravate the development of a systematic approach to transform recalcitrant strains. It might therefore be more promising to conclude the effort on *L. reuteri* CD037 and to turn towards other industrial *L. reuteri* isolates be it from animal or silage origin.



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## 7. Appendix

### 7.1. Abstract

Lactid acid bacteria are a highly heterogenous group of bacteria used in a variety of fields like food and feed fermentation, production of therapeutic proteins, and probiotics. Several lactic acid bacteria including strains of *L. reuteri* have been identified as true inhabitants of the gastrointestinal tract of humans, pig, and poultry, and are therefore promising candidates in industrial production of pro- and prebiotics.

Genetic engineering is used as a tool to meet industrial needs enabling introduction of new characteristics, modulation of gene expression, and more efficient secretion of desirable proteins. Electroporation is a common method to introduce foreign genes or promoters and is therefore crucial in genetic engineering. A vast number of fast and cost-effective electroporation protocols have been developed to allow for transformation of different microorganisms. Nevertheless protocols have to be optimised for each strain separately.

In this work we evaluated different established electrotransformation protocols for their suitability on our working strains belonging to the species *L. plantarum*, *L. reuteri*, *L. buchneri*, *L. lactis*, and *E. faecium*.

Furthermore we aimed at developing an electrotransformation protocol able to penetrate a specific industrial *L. reuteri* strain. We tested strategies including alterations of growth media, washing solutions, and electrical parameters in addition to already published electroporation methods proven to be successful in other *L. reuteri* strains.

Our strains differed highly in transformation efficiencies and optimal growth conditions. The chosen electroporation protocols varied in suitability depending on the choice of working strain and plasmid. Despite our efforts our *L. reuteri* strain remained reluctant to any transformation protocol which might be due to its pronounced agglutination and therefore inaccessible cell wall.

## 7.2. Zusammenfassung

Milchsäurebakterien finden ihre Anwendung in der Nahrungsmittelproduktion, Tierfutterfermentation, der Herstellung therapeutischer Proteine und Probiotika. Einige Spezies, wie etwa *L. reuteri*, werden aufgrund ihrer Fähigkeit den Gastrointestinaltrakt des Menschen und diverser Tiere zu besiedeln, als Kandidaten fuer die industrielle Produktion von Pro- und Präbiotika gehandelt.

Um die Charakteristika einzelner Stämme zu verbessern wird “genetic engineering” eingesetzt. Dies umfasst zum Beispiel das Einbringen fremder Gene und den Austausch von Promotoren zur kontrollierten Proteinproduktion und –sekretion. Eine erfolgreiche Methode hierfür ist die Elektrotransformation, die bereits für eine Vielzahl von Mikroorganismen angewendet wird. Die entsprechenden Protokolle müssen allerdings für jeden Stamm individuell optimiert werden.

Im Rahmen dieser Diplomarbeit wurden unterschiedliche Elektrotransformationsprotokolle an unseren industriellen Stämmen (*L. plantarum*, *L. reuteri*, *L. buchneri*, *L. lactis* und *E. faecium*) getestet.

Besonderes Augenmerk wurde auf die Entwicklung eines Transformationsprotokolls für einen bestimmten *L. reuteri* Stamm gelegt. Hierfür wurden die Zusammensetzung von Nähr- und Waschlösungen als auch elektrische Parameter variiert. Zusätzlich wurden Protokolle getestet, die bereits für andere *L. reuteri* Stämme erfolgreich eingesetzt werden.

Im Laufe der Versuche wurde gezeigt, dass die Wahl des richtigen Elektrotransformationsprotokolls entscheidend ist, um optimales Wachstum und Transformationseffizienzen zu gewährleisten. Trotz aller Bemühungen konnte der *L. reuteri* Stamm nicht transformiert werden. Dies könnte Folge seines agglutinierenden Wachstums und der damit erschwerten Zugänglichkeit der Zellwände sein.

## 7.3. Curriculum Vitae



### Europass Curriculum Vitae

#### Personal information



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Gender Female

#### Work experience

Dates	25/08/2011 →
Occupation or position held	Trainee
Main activities and responsibilities	Research on plasma polymerization and antibacterial coatings
Name and address of employer	Ian Wark Research Institute Mawson Lakes Campus, 5095 Adelaide (Australia)
Dates	01/02/2011 - 30/06/2011
Occupation or position held	Trainee
Main activities and responsibilities	Training in quality control and documentation in a GMP environment
Name and address of employer	Boehringer Ingelheim RCV Dr. Boehringer-Gasse 5-11, 1121 Vienna (Austria)
Dates	01/02/2010 - 31/12/2010
Occupation or position held	Lab technician
Main activities and responsibilities	Cultivation, electroporation and genetic engineering of lactic acid bacteria, research for diploma thesis
Name and address of employer	University of Natural Resources and Life Sciences, Institute of Applied Microbiology Muthgasse 18, 1190 Vienna (Austria)
Dates	12/01/2009 - 31/03/2009
Occupation or position held	Trainee
Main activities and responsibilities	Training in fermentation and development
Name and address of employer	Boehringer Ingelheim RCV Dr. Boehringer-Gasse 5-11, 1121 Vienna (Austria)

## Education and training

Dates 1/10/2002 - 30/12/2011  
 Title of qualification awarded  
 Principal subjects / occupational skills covered University Studies of Genetics, Microbiology, Chemistry and Anthropology  
 Name and type of organisation providing education and training University of Vienna  
 Level in national or international classification

Dates 14/09/2007 - 03/02/2008  
 Principal subjects / occupational skills covered Erasmus in Brussels including internship in a laboratory of the University of Brussels  
 Focus of research: cancer stem cells  
 Name and type of organisation providing education and training Vrije Universiteit Brussel  
 Brussels (Belgium)

## Personal skills and competences

Mother tongue(s) **German, Serbian**

Other language(s)

	Understanding				Speaking				Writing	
	Listening		Reading		Spoken interaction		Spoken production			
<b>English</b>	C1	Proficient user	C1	Proficient user	C1	Proficient user	C1	Proficient user	C1	Proficient user
<b>French</b>	B1	Independent user	B1	Independent user	A1	Basic user	A1	Basic user	A1	Basic user
<b>Italian</b>	B1	Independent user	B1	Independent user	A2	Basic user	A2	Basic user	A2	Basic user
<b>Dutch</b>	B1	Independent user	B2	Independent user	A2	Basic user	A2	Basic user	A1	Basic user

(\*) [Common European Framework of Reference \(CEF\) level](#)

Social skills and competences Eager to make new experiences, high sense of responsibility, team player, stress-resistant and open-minded

Computer skills and competences Good knowledge of Word and PowerPoint  
 Basic knowledge of Excel, SPSS, and Adobe Illustrator

Driving licence(s) B