Novel Bacteria, their Regulation and Safety Assessment

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Master's Thesis

submitted at the
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Department of Food Science and Technology
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October, 2014

Acknowledgments

I would like to acknowledge the support of my supervisors Prof. Seppo Salminen and Prof. Kneifel Wolfgang, who answered all of my questions as fast as they could. I am extremely grateful and indebted to them for their expert, sincere and valuable guidance. Thank you for writing me emails on Sunday, during the night and from the end of the world. Special thanks to the consulted experts worldwide who made a substantial contribution to this thesis and for their interesting work in general.

I take this opportunity to record my sincere thanks to all my professors and colleagues at the University of Natural Resources and Applied Life Sciences, the University of Copenhagen and the University of Vienna, who accompanied and supported me on my way. I would also like to acknowledge the support of AMA-Marketing, who gave me the opportunity to start working in addition to finishing my thesis.

To my mom and father, thank you for your support. Without you, I would not be the person I am. Thank you for your encouragement, your financial support during my studies and your deep and unconditional love. I also want to thank my sister, Valerie, who has always been the sunshine in my life. I wish you the best for your future life and your professional career. I also wish to express my sincere thanks to my deeply loved future husband, Christoph Stadlhofer, who always believed in me and encouraged me to continue writing. I am looking forward to spending my life with you.

Finally, I give my gratitude to all of the people who directly or indirectly have supported the development of my thesis. Outstanding thanks goes to Alexandra for proofreading and to Vanessa, Rafalea, Julia and Vivianne for the special moments full of love, fun and endless talks.

Abstract

This thesis focuses on the general regulation of novel bacteria, particularly probiotics that are intended for human consumption. Within the European Union, the regulation of novel bacteria remains challenging and expensive for applicants. The European regulatory framework contains grey zones in regards to genetically modified microorganism, substantial equivalence of related bacterial strains, and application of the European Qualified Presumption of Safety (QPS) system. Examples of these grey zones are discussed within this work.

The first discussed example is *Lactobacillus reuteri*, strain DSM 17938, and the regulation surrounding it as a genetically modified microorganism and conventional probiotic. Antibiotic resistance plasmids were removed from the mother strain (ATCC 55730) by techniques that are not considered as genetic modification by consulted experts and European law. It is likely that the mother and the daughter strains are substantially equivalent and therefore safe.

Another example of a novel bacteria is the commensal and abundant bacterium *Akkermansia muciniphila.* According to current scientific findings, this recently detected mucus degrader shows great potential at reducing health costs through disease prevention. The bacterium has not gained QPS or "novel food" status in terms of the Novel food Regulation (EC) No 258/97, but seems to be safe for future commercial production. Therefore, scientific work should focus on the functionality and importance of *Akkermansia* spp. in humans to facilitate the launch of novel probiotic formulations. Based on these examples, we can see that the repertoire and possibilities of proposed new probiotic candidates has not been exhausted.

Zusammenfassung

Diese Arbeit behandelt den gesetzlichen Regelungsrahmen von Novel Food (neuartige Lebensmittel), insbesondere von Probiotika. Die derzeitige Gesetzeslage in der Europäischen Union, betreffend der Zulassung neuartiger Bakterien ist langwierig und teuer für den Antragssteller. Weiteres umfasst das Europäische Regelwert einige Grauzonen, wie z.B. im Hinblick auf genetisch veränderte Mikroorganismen, substanzieller Äquivalenz von verwandten Bakterien und den Geltungsbereich des European Qualified Presumption of Safety (QPS: Qualifizierte Sicherheitsannahme) Konzepts. Diese Grauzonen werden anhand von Beispielen in dieser Arbeit diskutiert und erläutert.

Antibiotika resistenten Plasmide wurden dem ursprünglichen Mutter-Stamm *Lactobacillus reuteri* ATCC 55730 mit Hilfe von Techniken, die als nicht genetische Manipulation von konsultierten Experten und anhand der Europäischen Gesetzeslage eingestuft werden, entfernt. Der resultierende Tochter-Stamm *Lactobacillus reuteri* DSM 17938 ist entsprechend dem wissenschaftlichen Wissensstand sicher für den menschlichen Gebrauch, sowie mit dem Mutter-Stamm äquivalent.

Ein weiteres behandeltes Exempel stellt das probiotische und weit verbreitete Bakterium *Akkermansia muciniphila* dar. *A. muciniphila* zeigt großes Potential in der Prävention von Krankheiten und Krankheitssymptomen, sowie in der Senkung von Gesundheitskosten. Dennoch hat das Bakterium noch keinen QPS- oder „Novel Food"-Status erreicht. Zukünftige wissenschaftliche Arbeiten sollten die Funktionalität und das Potential von *Akkermansia* spp. untersuchen, um die Entwicklung neuer Märkte für probiotisches Produkte zu forcieren.

Lactobacillus reuteri DSM 17938 und *Akkermansia muciniphila* fungieren als Beispiele für das noch bei weitem nicht ausgeschöpfte Repertoire von neuartigen Probiotika.

Contents

Abbreviations

APHIS Animal Health and Inspection Service
BIOHAZ Panel on Biological Hazards
CFR Code of Federal Regulations
DG SANCO Directorate General for Health and Consumers
EC European Commission
EFSA European Food Safety Authority
EPA Environmental Protection Agency
EU European Union
EUCAST European Committee on Antimicrobial Susceptibility Testing
FAO Food and Agriculture Organization
FBO Food Business Operators
FEEDAP Panel on Additives and Products or Substances used in Animal Feed
FDA Food and Drug Administration
FD&C Act Federal Food Drug and Cosmetic Act
GFL General Food law (EU) No 178/2002
GM food Genetically Modified Food
GMM Genetically Modified Microorganism
GMM Directive Directive 2009/41 *on the contained use of genetically modified microorganisms*
GMM Guidance Guidance *on the Risk Assessment of Genetically Modified Microorganisms and their Products Intended For Food and Feed Use*
GMO Genetically Modified Organism

GMOs Directive Directive 2001/18/EEC *on the deliberate release into the environment of genetically modified organisms repealing Council Directive 90/220/EEC*

GMOs Regulation Regulation (EC) No 1829/2003 *on genetically modified food and feed*

GRAS Generally Recognized as Safe

MIC Minimum Inhibitory Concentrations

MUC Mucus-Binding-Protein

NCA National Competent Authority

NDA Panel on Dietetic Products, Nutrition and Allergies

NIH Guidelines National Institute of Health Guidelines for Research Involving Recombinant Synthetic Nucleic Molecules

NFR Novel Food Regulation (EC) No 258/97 *concerning novel foods and novel food ingredients*

Pbps Penicillin-Binding-Proteins

PCR Polymerase chain reaction

QPS Qualified Presumption of Safety

USA United States of America

repPCR Repetitive Extragenic Palindromic PCR

rRNA Ribosomal Ribonucleic Acid

WHO World Health Organization

1. Preface

The human intestinal tract is colonized by a diverse biota of up to 500 bacterial species. Each individual possesses his/her own enteric fingerprint, consisting of a specific intestinal bacterial pattern (Quigley 2010). It is important to stress that the real dimensions of the human microbiota are still a topic of research. Nevertheless, modern technological achievements, based on *16S ribosomal ribonucleic acid sequences* (rRNA) and molecular techniques as real-time *polymerase chain reaction* (PCR), clone libraries and metagenomics, have been beneficial for deciphering the actual diversity of the intestinal ecosystem. Notwithstanding, the cultivation of bacterial sequences and the sufficient safety assessment of newly described strains remain challenging (Duncan et al. 2007; Hattori and Todd 2009; Tu et al. 2014).

For thousands of years, microorganisms, especially *Lactobacillus* spp. and *Bifidobacterium* spp., have been used in food production, with a long history of safe application (Caplice and Fitzgerald 1999; FAO/WHO 2002). It is feasible that some viable bacteria "when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002, p. 8). Recently, a non-profit international expert group redefined the classification and definition of probiotics. This group also aimed to correct some minor grammatical errors (Hill et al 2014).

Physicians and the food industry have supported those beneficial commensal bacteria as probiotics (FAO/WHO 2001). Although probiotics generally enjoy a well-established safety record, some features are similar to those described in known pathogens and can lead to opportunistic effects in susceptible individuals (Hattori and Todd 2009; Quigley 2010). The safety of probiotics and the necessity for harmonized assessment guidelines have been a subject of discussion by several institutions worldwide (European Commission 2003a; FAO/WHO 2002). Different approaches were published over time, such as the *General Recognized as Safe* (GRAS) notification system used in the *United States of America* (USA) or the relatively new *Qualified Presumption of Safety* (QPS) scheme adopted by the European Commission (EC) and the European Food Safety Authority (EFSA). The latter approach focuses particularly on the body of knowledge and potential antibiotic resistances of the microorganism of interest (EFSA 2005a).

The regulation of microorganism has also become increasingly important due to the diversity of possible applications, such as resistance plasmid-curing techniques. Thus, it is of importance to acknowledge if the modified "derivative" strain should automatically gain the same safety status as its "parent" strain with respect to substantial equivalence (Rosander et al. 2008). Beside the QPS scheme, the *Novel food Regulation* (EC) No 258/97 (NFR) in the *European Union* (EU) plays an important role in assessing the safety of modified "old friends" and recently introduced bacteria (European Commission 1997c; European Commission 2003a).

2. Introduction and Fundamental Aspects

Various approaches for the regulation of microorganisms intended for consumption by humans and animals exist worldwide. The EU and USA are the most prominent regulatory powers influencing global standards of food and feed products. Their decisions and regulations set examples for all other countries, including the growing Asian market (Wessels 2012). Although the technologies and microorganisms applied in the American and European food sectors are almost identical, their regulative approaches are diverse. Reasons behind the differences are explained by divergent regulatory developments and industry or consumer interests (Lynch and Vogel 2001). Furthermore, the European Union has a dynamic character and a short history in shared regulatory developments when compared to the USA. Additionally, the number of Member States is continuously increasing.

It has been shown that European citizens tend to prefer conventional and minimally processed food products, whereas US citizens accept new technologies faster. This has hindered the European development of harmonized food regulation and has allowed the USA a market benefit through earlier introduction of new technologies (Echols 1998). When one compares the European and American approaches to risk, it is important to consider their cultural and historical backgrounds (Lynch and Vogel 2001; Wessels 2012, p.12). In contrast to the relatively newly united European Union, the unification of the modern USA occurred in 1776 with the *Declaration of Independence* and indicates a long history of common centralized regulation providing free transport of goods in a manner similar to that of European regulation recently (Bilhartz and Elliott 2007). US food and drug administration (FDA) recognised the importance of regulating food safety and food additives with their emergence at the beginning of the 20th century (FDA 2011). At that time, a united Europe was still in its infancy.

2.1. European and US Food Law

Similar to Europe, the USA regulated food at a state level until the 19th century, covering a wide range of heterogeneous legislation. With the emergence of new technologies, the detection of food adulteration more and more became challenging, leading to the development of single food law approaches (Law 2004). In 1906, the first US federal food law was enacted. The *US Food and Drugs Act* constitutes the cornerstone of global

consumer protection and health laws (U.S. Congress 1906). The *Bureau of chemistry*, followed by the *Food and Drug Administration* (FDA) and *Federal Security Agency*, enforced the Act.

In 1938, the *Federal Food Drug and Cosmetic Act* (FD&C Act) replaced the original law, as a toxic elixir resulted in 107 deaths. Consequently, the FDA gained influence and introduced the first pre-market safety approval program for novel drugs. Further incidents with toxic substances corresponded with intensified laws to test the effectiveness of new ingredients. The *Food Additives Amendment* was added to the FD&C Act in 1958 and outlined the definition of 'food additives' for the first time (see Chapter 4). Currently, drugs, food and feed are still regulated according to the FD&C Act in the USA (Gaynor 2006; FDA 2011; Law 2004). As the executive body, the FDA controls approximately 80% of the food market. As part of the FDA, the *Center for Food Safety and Applied Nutrition* is now accountable for the safety of food and cosmetics, particularly food additives, biotechnology, foodborne contaminants and labelling (Gaynor 2006).

The European food production market has changed dramatically since 1990. Increasing cross-border trade, emerging technologies and mass-production were accompanied by several serious food crises. Subsequently, the European populations' confidence has been shattered since dioxin scandals and zoonotic diseases spread throughout Europe (Birmingham 2000; Erickson 1999; Kupferschmidt 2011). The fear of bacterial contamination in animals, transmitted through the food chain to humans, seems to still be part of the European mentality. Additionally, new technology used in food products, especially genetical modification and nanotechnology, have caused doubts and insecurity among many people (Wessels 2012). The establishment of the *Treaty of Maastricht* and the *Treaty of Lisbon* in 2009 have formed an important basis for the development of regulation approaches for beneficial microorganisms (OJEU 2012; OJEC 1992; Wessels 2012). Due to the need for harmonized food regulation, the *White Paper on Food Safety* was constituted and is considered the foundation for the *General Food law* (GFL) Regulation (EU) No 178/2002 (European Commission 2000b; European Commission 2002d; van der Meulen and van der Velde 2011).

In the GFL, two principals have been crucial for the harmonization of law on beneficial microorganism. First, the general principles on food safety, introduced in Chapter III, announced the *European Food Safety Authority* (EFSA) as an independent advisory and

risk assessment institution. Second, the European members are represented, in terms of food, by the *Directorate General for Health and Consumers* (DG SANCO) as part of the Commission and its standing committees (e.g. *Standing Committee on the Food Chain and Animal Health*) (von Wright 2012). Prior to the introduction of the GFL, Member States established their own food safety regulations. The Preamble 30 of the GFL insists on the importance of barrier free trade between member countries, and therefore requires all European *food business operators* (FBO) to comply with one general food law (European Commission 2002d).

Food law in the EU is, in most cases, laid down as regulation and directly applicable with no leeway in decision-making for Member States. This approach provides two advantages: no room for interpretation of food safety and relatively fast implementation of new laws (OJEU 2006; Wessels 2010; van der Meulen and van der Velde 2011). Consequently, the responsibility for beneficial microorganism has been transferred from specific Member States' authorities to the comprising EU authority (Wessel 2010).

2.2. Motives for Regulating Probiotics

Products containing starter cultures have been used to improve intestinal microbiota long before the term "probiotic" was established. The regular consumption of fermented dairy products seems to have no apparent adverse health effects. Contrary to starter bacteria are probiotic strains, mostly lactic acid bacteria and *Bifidobacterium* ssp., able to remain viable during transit through the gastrointestinal tract. They possess further characteristics, which contribute to the restoration of intestinal microbial balance, such as competitive adherence (Derrien et al. 2004; Donohue and Salminen 1996; Tuomola et al. 2001).

The list of reported strain-specific health effects is comprehensive and varies from improving gastrointestinal discomfort, immune modulation, and reducing relapse of inflammation, to obesity prevention (Böhm and Kruis, 2006; Everard et al. 2011; 2012; Reid 1999). Nevertheless, the exact health effects employed by many probiotics are not fully understood. The best or the best documented evidence is allocated to therapy of acute diarrhoea (or acute gastroenteritis in infants and children), but more data is required for understanding the potential in the reduction of risk of antibiotic associated diarrhoea and side effects (Williams 2010).

Regardless, the given effects appear to be strain specific and might not be transferable to others. Most of the effects are dose related, since a minimum number of 10^8 bacteria must be administered (Verna and Lucak 2010; Williams 2010). With the rising amount of promoted products doubts have arisen concerning quality assurance, scientific evidence of health effects and safety of probiotic products (Reid 1999). In contrast to certain products, where the lack of quality and safety is obvious, are drugs and microorganism containing products which are difficult to assess by consumers (Law 2004).

The involvement of known probiotics in bacteraemia and infections is known, although the events are extremely rare considering the use of probiotics in foods and occur predominantly in immunocompromised patients with ongoing serious underlying disease (Antony et al. 1996; Antony 2000; Bernardeau et al. 2008; Salminen et al. 2006). However, novel strains might host pathogenic or other detrimental properties and raise the need for more extensive safety assessment. For instance, translocation from the intestinal tract to "sterile" organs is undesirable and followed by severe consequences for the host. Additionally, adhesion to mucous cells is an expected attribute of probiotics but can also be involved in invasive pathogenic behaviour (Derrien et al. 2004; Donohue and Salminen 1996; Tuomola et al. 2001). Nevertheless, mucin-degrading bacteria have recently been under investigation as potential probiotics due to resulting selective growth and impact on the intestinal epithelium (Derrien et al. 2010).

The presence of antibiotic resistance genes in foods associated with bacteria and probiotics is a current topic of great concern — but highly strain dependent (Salminen et al. 2006). One has to distinguish between acquired and intrinsic resistances. Acquired resistances can be caused by acquired genes or gene mutations and are only found in a limited number of species related strains (EFSA 2008b). Potential genes can potentially be transferred to the indigenous microbiota or pathogens by horizontal gene transfer, resulting in a decline of available medical treatments (Egervärn et al. 2009b). The transfer itself is related to the genetic location of the resistance, especially to mobile elements such as plasmid, transposon and integron (EFSA 2008b). However, according to the EFSA, there are strains with resistances through chromosomal mutations or non-transmissible intrinsic features. Such resistances are inherent to all strains of a species and are safe for feed, as the risk of horizontal gene transfer can be avoided (EFSA 2005b).

Consequently, the absence of transmissible resistance genes in each newly identified or characterised strain is an important property to evaluate (Egervärn et al. 2009b; Klare et al. 2007). The quantitative determination of antibiotic *minimum inhibitory concentrations* (MIC) and their breakpoints must be performed, together with an analysis of the genetic origin of the resistances (EFSA 2008b). To distinguish susceptible strains from resistance strains or strains with intrinsic resistances ("wild types"), knowledge of their MICs is necessary. MIC is defined as the lowest concentration of an antibiotic to inhibit the bacteriums' growth. Values above the MIC breakpoint act as indicators of special resistances (Egervärn 2009a). The *European Committee on Antimicrobial Susceptibility Testing* (EUCAST) deals with breakpoints and susceptibility testing, providing helpful information about MIC distributions and breakpoints (EUCAST 2014).

Besides resistance, some species of *Lactobacillus* are linked to biogenic amines production and other unfavourable properties, such as enzymatic activities, which are important to be excluded (Bernardeau et al. 2006).

2.3. From the Strain to the Novel Probiotic

It is of special concern to this work to understand the basic steps of efficacy substantiation of probiotics for human consumption. Figure 1 summarises the different phases necessary to approve a bacterial strain as a probiotic.

Everything begins with an appropriate and specific bacterial strain. Fermented dairy products have been a rewarding source of valuable bacteria for a long time. The intestinal microbiota of humans, animals, and even breast milk also seem to host several beneficial strains. Therefore, many probiotics available on the market are derived from human microbiota (Derrien et al. 2004; Fontana et al. 2013; Petrof 2009; Sinkiewicz and Ljunggren 2008).

When it comes to the isolation, identification and characterisation of strains, the regulation appears to be challenging, as many companies use undefined multi-strain cultures and questionable claims (EFSA 2005a). Based on an investigation, 63% of tested probiotic products in the United Kingdom were insufficient (Hamilton-Miller and Shah 2002). Either the promoted composition differed from the existing one, or the strains were labelled in an incorrect or misleading way. Additionally, the expected viability of bacteria was not evident in many products (Hamilton-Miller and Shah 2002). To clarify the *Food*

and Agriculture Organization (FAO) and *World Health Organization* (WHO) working group established new guidelines in 2002 for the substantiation of probiotics in food, as listed below:

1. **Strain identification by phenotypic and genotypic techniques** (genus, species, strain) and identification of the strain-specific effects, using state of the art methodology and methods (e.g. 16S RNA gene analysis, DNA-DNA hybridization etc.) combined with phenotypic testing schemes (e.g. fermentation of sugars).
2. **Tests for functional characterization** correlating with **in vitro, in vivo and human studies phase 1 tests** which are focused on safety. It is recommended that even probiotics considered as GRAS (see Chapter 4.1) should be tested for metabolic harmful products (D-Lactate, bile salt deconjugation), as well as antibiotic resistances, adverse side effects in humans and epidemiological surveillance, haemolytic activity, antimicrobial activity and toxin production. Optimally the results should be proven in immune-comprised animals and be target specific. In vitro tests should include gastric activity and bile acid resistances, mucous adherence, antimicrobial activity and competitive behaviour against pathogens, hydrolyse of bile salt and if necessary resistances to spermicides.
3. **Animal studies and human studies phase 2** accompanied by double blind, randomized, placebo-controlled design to test the efficacy of the strain and if necessary an independent control study.
4. **Human studies phase 3** to test the effectiveness of treatments and if necessary post-market surveillance studies to test long-term effects.
5. Guidelines on product labelling, including contents (Genus, species and strain information), number of minimum viable bacteria, shelf life, appropriate storage conditions, approved health claims and company contact information.

All guidelines, findings and adverse effects must be published in a peer-reviewed scientific journal (Anadòn et al. 2014, p.91; FAO/WHO 2002).

Figure 1 illustrates the above-mentioned five points in form of a flow chart. One of the most important points represents the exact determination of strain identity, which plays a crucial part in the systems and is elaborated upon in Chapter 4. In the EU, novel bacteria are covered according to Regulation (EC) No 258/97 (see Chapter 4.3.) and, in case of proposed health claims, according to Regulation (EC) No 1924/2006 (European Commission 1997c; 2006).

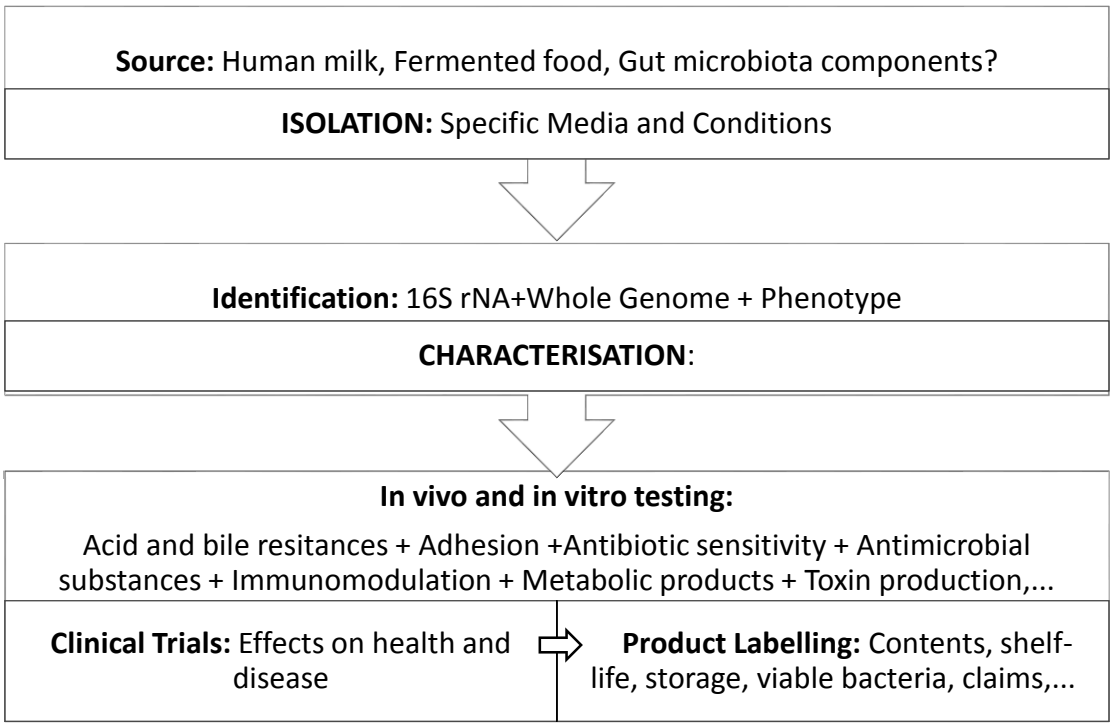

Figure 1: Flow diagram including the basic steps for the approval of novel probiotics according FAO/WHO Guidelines 2002 (FAO/WHO 2002)

3. Purpose

The aim of this work is to elucidate European regulatory grey-zones in terms of potential novel and modified probiotics. This work is divided into three parts. The first part reviews the general regulatory framework in the EU and USA. The first research question is addressed in Chapter 5, where the safety of two *Lactobacillus reuteri* strains is assessed according to the European *Qualified Presumption of Safety* system (QPS). Antibiotic resistance plasmids have been removed of strain ATCC 55730 by a technique where the actual genome has not been touched, resulting in the daughter strain DSM 17938. Nobody before has questioned if the plasmid removing technique might lead to any unfavourable effects, or what has to be considered in terms of regulation. *L. reuteri* strain DSM 17938 is part of a regulatory grey area, as it is neither genetically modified in a traditional manner, nor "untouched" anymore. This work questions the safety of strain DSM 17938 and the regulation surrounding it as a potential *genetically modified microorganism* (GMM) and as a conventional probiotic. To clarify if plasmid-curing techniques result in GMMs, experts were consulted for their opinions. This is summarized in Chapter 5.3.

Part three deals with the recently explored intestinal bacterium, *Akkermansia muciniphila*, and its regulation and safety as a potential novel bacterium according to Novel Food Regulation (EC) No 258/97. Data and information dealing with this mucin degrader are limited. Consequently, the aim of this work is to utilize available literature to discuss which data are still missing to fulfil a respective novel food authorisation.

4. Regulation of Probiotics in the EU and USA

Unlike the complex EU regulation of microorganism, the US solution is simplified due to the fact that feed underlies the same laws as food. In the States, microorganisms intentionally added to food are termed as "food additives" or as "dietary supplements", with one exclusion: if the product or the microorganism is linked to treatment or prevention of disease, it is characterised as a drug and requires detailed safety assessment in a manner similar to other pharmaceuticals. In the case of a named dietary supplement, the probiotic product is recognised as a food and therefore regulated by the FDA's *Center for Food Safety and Applied Nutrition*. No pre-market approval for dietary supplements is required in the US, unless the substance provides no history of safe use and has been introduced after 1994. In such a case, the Agency must be notified (von Wright et al. 2004; FDA 2014a) with a so-called GRAS notification including an expert assessment of the safety of the product. Supplements shall not be confused with approved GRAS microorganisms for conventional food, as dietary supplements have their "own grandfather list" according to the *Code of Federal Regulations* 182 (CFR) (FDA 2013b). Importantly, it is not allowed to add supplements to conventional products unless they have been cleared through the GRAS notification system (Israelsen 2002).

In contrast, the European regulatory approach for microorganisms intended for food is unaccomplished, while that for feed is well-defined (European Commission 2009b). Beneficial microorganisms for food can be either additives or ingredients, whereas probiotics are usually not recognized as additives (SANCO 2006). Nevertheless, probiotic bacteria for food are authorized according to EU law under three explicit categories: genetically modified microorganism, microorganisms considered as novel foods and microorganisms with health claims associated with human probiotics (von Wright et al. 2004). Probiotics on the market have to fulfil at least one of the three categories and conform to the respective regulation (Anadòn et al. 2014). Although they are widely used, no specific European legislation for probiotics as food supplements exists (Directive 2002/46/EC) (European Commission 2002c).

The substantiation of health claims from probiotics remains a great challenge for both the regulatory agencies (EFSA) and the industry. However, this topic will not be elaborated upon further in this work (Anadòn et al. 2014). If probiotics are promoted as

drugs, they must undergo the required drug registration process, similar as in the USA (Saxelin 2008). The QPS, a new harmonized safety assessment approach for microorganisms established by the EFSA in 2007, has been inspired by the GRAS system in the USA. Both systems, GRAS and QPS, are supposed to make regulation more straightforward (Sundh et al. 2012). Therefore, microorganisms accompanied by a sufficient history of use and a complete fulfilment of requirements according to the QPS scheme can be approved as QPS organisms (EFSA 2007). Novel microorganisms which are taxonomically undefined, without long-term experience and lacking substantial equivalence, are regulated by the NFR and require a full-safety assessment. Where the identity of the microorganism can be defined, the QPS system may be applicable (EFSA 2005a; European Commission 1997b; c).

4.1. The US Approach: Generally Recognised as Safe

The FDA and its *Division of Biotechnology and GRAS Notice Review* are the regulative authorities behind the assessment of novel or GRAS microorganisms (Wessels et al. 2004). According to US regulation, microorganisms intended for food and feed are either food additives or "substances that are GRAS under condition of their intended use and do not require premarket approval by FDA*"* (Gaynor 2005/2006, p.1).

Contrary to this definition, all substances that are deliberately added to food comprise food additives according to sections 201(s) and 409 of the FD&C Act. Pre-market approval, including safety documentation, is required. Only three dried microorganism are listed as food additives in the USA so far: *Saccharomyces cerevisiae*, *Candida utilis* and *Saccharomyces fragilis.*

Two possibilities exist for microorganisms to receive GRAS status: 1) documented and generally agreed upon scientific evidence and 2) a known history of safe use by a significant population prior to 1 January 1958— the so called "grandfather rule" (FDA 2004; 2011; 2013a;b; Gaynor 2005/2006). The intended use plays a crucial role in GRAS approval. The same strain can achieve GRAS status in a specific diary product, but is not permissible in others. Therefore, GRAS status is closely linked to usage and expert evaluation of the safety (FDA 2004).

Interestingly, the onus of proof lies with the respective company and not with the federal food authority. A qualified independent panel of experts must provide evidence

on behalf of the company. Normally it is in the best interest of producers not to jeopardize consumers' safety, as a subsequent lawsuit can be a tremendous liability for reputation and finances. It can be assumed that the producers of probiotics are highly motivated in testing the desired strain as comprehensively as possible (Wessels 2012). The FDA 'only' assesses the applicant's submitted documents and either grants or does not approve GRAS status. However, it is not necessary to ask the FDA when applying for a strain to a product, as pre-market approval is not required. By law, the FDA does not even need to be informed. The responsibility lies completely with the company, as the submission of GRAS notifications is voluntary. However, usually it is in the interest of the company to verify the GRAS notification status as suggested by the regulation. In comparison to food additives, data about GRAS substances are widespread, generally accessible and scientifically sound. Knowledge about food additives is normally the property of the company and treated confidentially (FDA 2004). It is therefore discussable, whether the expenditure of scientific proof is proportionate to the benefits of granted GRAS status for companies. However, in legal terms and in reference to the responsibility of the product including economical and legal responsibility, it is of great importance to the companies to have a GRAS status affirmed.

Moreover, for most substances an approved GRAS status can open up the market as a "novel food ingredient or a food supplement ingredient" under European law (Israelsen 2002). Additionally, if a company introduces a product on their own behalf, which causes a food safety incident, the company has no support from the FDA and is completely liable. In case of a food safety incident involving a GRAS approved strain, the FDA theoretically shares responsibility with the company, as the agency agreed on the submitted safety assessment before.

Likewise, the FDA profits from the GRAS evaluation system. In comparison to a food additive the efforts and expenses of a GRAS assessment are minimal, since the company does the administrative and scientific work. Food additives require toxicology and efficacy studies carried out by the FDA and therefore need more effort and resources (Gaynor 2005/2006; Wessels et al. 2004).

The origin of this convenient system can be traced back to President Nixon. In 1960, President Nixon asked the FDA to update the safety of GRAS due to uncertainties concerning cyclamate salts. The result was a laborious and resource-intensive re-

examination of petitioned substances supposed to be GRAS ("GRAS Affirmation"). In 1997, the FDA laid down a new rule, the *GRAS Notification Program* (the GRAS proposal; 62 FR 18938). This program made it possible for volunteers to notify the FDA if any substances could be considered GRAS according to their determination, moving the legal burden of proof for safety towards companies (Gaynor 2005/2006). The notifier must submit a portrayal of the substance (description, properties, identity), the intended use, chemical, toxicological and microbiological data and determining criterion: history of safe use or scientific evidence. Finally, an objective discussion of results shall be added to the application. The FDA itself has 90 days for responding and assessing whether the received notice is adequate for GRAS status (Department of Health and Human Service/FDA 1997; FDA 2013c). The received GRAS notifications, FDA responses and additional information are summarized online in the *GRAS Notice Inventory* (FDA 2014b).

It must be emphasized that the GRAS list has an open character, as it would be impossible to add all substances with GRAS features. However, microorganisms can be GRAS without being listed online when all data points to that conclusion (Anadòn et al. 2014, p.91). Any microorganism or substance not GRAS approved must go through a pre-market safety assessment. The company decides whether to apply for a food additive or carry out a GRAS self-affirmation. However, in terms of probiotics, the industry will focus less on additives as they do not implicate the favoured characteristics (see CFR 182 Subparts c, d, e, g, h) (Israelsen 2002).

4.2. The EU Approach: Qualified Presumption of Safety

One of the pioneers of European regulation was Denmark with its regulated pre-market approval system for new cultures, resulting in a detailed list of microorganisms (Danish Veterinary and Food Administration 2013). For years the Union only concentrated on the regulation and safety of feed probiotics and did not consider probiotics for food to the same extent (OJEC 1993). Due to different frameworks for food and feed, bacteria added to feed were subject to a comprehensive assessment procedure but did not require notification when added to food. Antibiotic resistance properties in bacterial strains were interestingly not a topic of concern in feed if the respective strain enjoyed a traditional status of use (EFSA 2005a).

Finally, in 2003, a working group established by three committees of DG SANCO drafted a proposal for *the safety assessment of microorganism used in feed/food and feed/food products* (European Commission 2003a). Newly established EFSA adopted the idea and officially implemented it in 2007 with the *Panel on Additives and Products or Substances used in Animal Feed* (FEEDAP) as its' primary client (EFSA 2007). Since their first review in 2007, the *Panel on Biological Hazards* (BIOHAZ) has published annual revision of the QPS list (EFSA 2008b; 2009b, 210-2014). Each review includes an update of already covered *taxonomic units* with respect to new technologies and the assessment of both (Leuschner et al. 2010).

The main purpose of the approach is to harmonize and simplify the assessment process for novel microorganism added to food or feed at a species level, with due regard to European risk perception. Focusing on "more harmful subjects", the EFSA is able to save both human and economic resources (European Commission 2003a; Leuschner et al. 2010). Different from the GRAS list, the scope of QPS is limited to microorganisms and their metabolites but has potential to be extended (EFSA 2007). The whole concept has been influenced by the flexibility of the US GRAS system, but is not identical since the focus lies on European issues. For instance, acquired antibiotic resistance factors are an ongoing topic of discussion in the Union, but not perceived as a major problem in the USA. Similar to GRAS, the QPS scheme is described as *"*an assumption based on reasonable evidence*"* with the aim of prioritizing EFSA resources (European Commission 2003a). Microorganisms with a well-established history of use do not require a comprehensive safety assessment, since it seems to be more important to focus on acquired antibiotic resistance and virulence factors. Any safety concerns must be excluded and elaborated. According to the first proposal by the Commission, the QPS is defined as a pure assessment approach and provides neither a legal status nor any benefits for the notifier. Unlike the GRAS system, the primary burden of proof lies on the EFSA with the possibility for notifiers to add additional information and submit proposals.

The whole system is based on four pillars:

1) “Taxonomic identification” of the highest definable unit by state-of-the-art methods

2) “The body of knowledge”: a sufficient description of history of use and level of exposure, safety data in scientific literature, industrial applications, ecological impact and clinical data

3) “Exclusion of pathogenicity”

4) “End use” description

The last point, “end use” of the microorganism, is crucial because it influences the “body of knowledge” and “identity” in their applicability. Depending on the assessment of live, dead or production microorganism, different outcomes might be relevant (European Commission 2003a; 2005a; Leuscher et al. 2010).

The determination of identity (genus, species, and subspecies) is the key-piece of the whole assessment and decides if the microorganism is suitable for QPS. If it is impossible to identify the taxonomic unit or link it to any known species, QPS status is denied. A wealth of taxonomic data is available through molecular techniques. Therefore, distinguishing between “What is need to know” and “What is nice to know” is important for maintaining simplicity of the system. The “body of knowledge” provides the second crucial point and correlates with taxonomic data, followed by determining if the “proposed use” of the microorganism is novel or traditional (Bergmans 2012; EFSA 2005a). In case of a novel application, the microorganism must be regulated according to the NFR (EC/258/97).

The absence of pathogenic and virulence properties among the assessed taxonomic unit (on a species or genus level) must be proven through clinical data and scientific literature. In case of existing pathogens, it is important to consider if the knowledge is sufficient to describe and subsequently exclude those. If an exclusion is not possible, the species seems to not be suitable. Other aspects, if relevant, should be included in the assessment and profoundly described (e.g. release in the environment and antimicrobial resistances) (Anadòn et al. 2014; Bergmans 2012; EFSA 2005a).

A lack of “history of safe use” must not be stated as excluding criteria as long as all other requirements are sufficiently fulfilled. However, “long history of safe use” does not automatically guarantee safety from every point of view. Any safety aspects possibly

affected adversely by processing steps and other ingredients are not a topic of concern for the QPS system (European Commission 2003a; 2005a; Leuscher et al. 2010).

Opportunistic bacteria, which have been linked to infections in immune-deficient people, are not necessarily excluded from the list when adequately described and documented. However, pathogenic or toxin producing strains must be completely omitted from the list which does not necessarily apply to the whole "taxonomic unit" (European Commission 2003a). In case of genetically modified microorganisms (GMM), the respective change of phenotype and genetic information does not affect the assessment process, as other regulations are accountable (see Chapter 4.4) (European Commission 1998; 2001).

In particular, the QPS approach focuses on the absence of acquired antimicrobial resistances and virulence factors relevant for the efficacy of medicine. In case of production strains which are not detectable in the end product, resistance properties might be negligible. In essence, applied bacteria should not produce substances, which are similar to antibiotics for human or animal treatments.

Already approved strains only require a registration when the production conditions have been adopted (European Commission 2003a), whereas those who failed to fulfil the QPS requirements must undergo a full safety assessment (Leuschner et al. 2010).

Currently, the list includes traditional microorganism and those with a novel application. The majority consists of gram-positive and non-sporulating bacteria, such as *Bifidobacterium, Corynebacterium, Lactobacillus, Leuconostoc, Pedicoccus, Propionibacterium, Oenococcus oeni* and *Streptococcus termophilus.*

Most of the listed bacteria do not require any revision in respect to their QPS status, with the exception of *Enterococcus faecium,* which is only approved as a feed additive and not considered a "safe" bacterium. *Enterococcus* strains, which carry antibiotic resistances, are frequently responsible for nosocomial infections and the distinction between virulent and non-virulent strains remains problematic (EFSA 2013). The majority of the recommended gram-positive spore forming bacteria belongs to the genus *Bacillus,* and only those that lack of toxigenic activity. All mentioned bacteria must be free of transferable antibiotic resistances or, if unavoidable, absent as a viable form in the end product (Leuschner et al. 2010).

In the case of gram-negative bacteria, for example *Escherichia coli* and *Serratia rubidae,* the evidence has not been sufficient to date. Although *E. coli* possesses a long history of safe use and a sufficient body of knowledge, strains are also responsible for a number of infections in humans. The exclusion of all pathogenic *E.coli* strains would not contribute to a simplified assessment scheme. All other assessed gram-negative bacteria also lack in safety and sufficient body of knowledge (EFSA 2009b). *Gluconobacter oxydans,* for the intended use of vitamin utilization, is the only gram-negative bacterium recommended for the QPS list (EFSA 2013). Besides bacteria, yeasts, filamentous fungi, bacteriophages, as well as plant and insect viruses are part of the annual QPS list update, highlighting the potential for QPS assessment extensions (EFSA 2013). Furthermore, the addition of botanicals to the QPS list has recently been discussed (EFSA 2014).

Taken together, the approach has been gratefully accepted among notifiers and EFSA, as it allows for the possibility of concentrating on essential problems, reduces the number of vivo studies, decreases the use of animal studies, strengthens consumer faith and encourages the development of new products.

4.3. European Novel Food Regulation (EC) No 258/97

With the exception of two regulations, the Novel Food Regulation (EC) No 258/97 and the Health Claims Regulation (EC) No 1924/2006/EC, microorganisms and specifically probiotics are not subject to the European regulative framework (European Commission 1997c; 2006). The NFR was established to ease public concerns about emerging technologies within the food industry and, subsequently, "to protect the functioning of the internal market within the Community*" (*European Commission 2002b). The main scope is the same as laid down in Regulation 178/2002/EC and shall protect the consumer against danger, misleading and nutritional disadvantages (European Commission 1997c; 2002d). The current NFR comprises Regulation (EC) No 258/97 and Commission Regulation (EC) No 1852/2001 concerning information management (European Commission 1997c; 2001b).

The NFR was laid down to cover the safety of food and food ingredients which have not been introduced to the market to a "significant degree" prior to 15 May 1997. These novel ingredients without a "history of safe use" require pre-market approval to guarantee their safety. The NFR seems to cover a wide range of products and substances.

Nevertheless, *genetically modified organisms* (GMO) were excluded and have been separately regulated since 2003 (see Chapter 4.4). Results of conventional breeding, new combinations of ingredients, food additives, flavours and extracting solvents are exempt from the NFR as well. A separate authorisation process under the NFR is necessary to approve substances and products that fall under dietary supplements for food.

"Novel Food" is defined in different ways. It can either be a product or substance that has previously not existed worldwide, or merely in the EU, and is therefore perceived as "exotic". Moreover, it can be produced by an innovative new technology with a direct impact on the properties of the food.

In terms of probiotics, only the second category of novel foods mentioned in Article 1(2) is relevant: "(d) foods and food ingredients consisting of or isolated from micro-organisms, fungi or algae" (European Commission 1997c).

Substances and products that are not defined as novel must have been consumed within Member States to a significant degree before 15 May 1995. Occasionally criticised are the possibly broad interpretations of "significant degree" or "novel food" (European Commission 1997b; van der Meulen and van der Velde 2011). According to a discussion paper by the European Parliament and Council, "significant degree" can be interpreted as generally available in food stores in at least one Member State (European Commission 2002b).

In case of microorganism, the NFR does not define which taxonomic level "novelty" applies to (genus, species or strain). To date, no microorganism itself has been approved as a novel organism according to the NFR. Only products from microorganisms are authorised, for example, in Commission Decision 2009/345/EC Vitamin K2 (menaquinone) was utilised from *Bacillus subtilis.* The status of a limited number of products containing bacteria, such as *Clostridium butyricum*, are still outstanding (European Commission n.d.b). *Cl. butyricum*, as a probiotic food supplement, is the first live bacterium that takes part in the NFR authorisation process since failing QPS status (Advisory Committee on Novel Foods and Processes/FSA 2013).

It is possible to achieve novel food status in two ways: through an application or through a simplified procedure called the "notification". The pre-market assessment procedure first includes the Member State's *national competent authority* (NCA) and second the Commission, including its' *Standing Committee on the Food Chain and*

Animal Health. In the case of a regular application, the NCA first receives the proposal, proves novel status, and decides whether additional measures are necessary. The initial assessment report is then forwarded to the Commission, which informs other Member States. If all parties agree and no Member State raises objection, the company is allowed to place the product on the European market. If there are any uncertainties about whether a product is novel or not, the *Novel Food Working Group* can be consulted. The group consists of experts from Member States and officials from the European Commission. In the future risk assessment, carried out by the EFSA, will gain more and more importance (European Commission 1997a; c; 2008).

A notification is based on a similarity to already existing products or ingredients: the so-called "substantial equivalence" with respect to composition, nutritional value, metabolism, intended use and level of undesirable substances. This leads to a simplified assessment procedure. If it is agreed that a novel product is substantially equivalent to a traditional one, or an already approved novel food, the same safety status can be assumed. In case of no substantial equivalence the product or ingredient is not unsafe, but requires a safety assessment linked to its' unique characteristics (European Commission 1997a;c).The applicant is obliged to inform the European Commission when he/she has brought the product to market and the Commission hereafter informs Member States (European Commission 1997c).

Two possibilities exist for achieving substantial equivalence. First, the product provides either generally recognised scientific evidence or a scientifically proven safety status by the NCA. Second, the given authorisation in both application procedures is limited to the approved product and defines its condition of use. The whole novel food application procedure is illustrated in Figure 2.

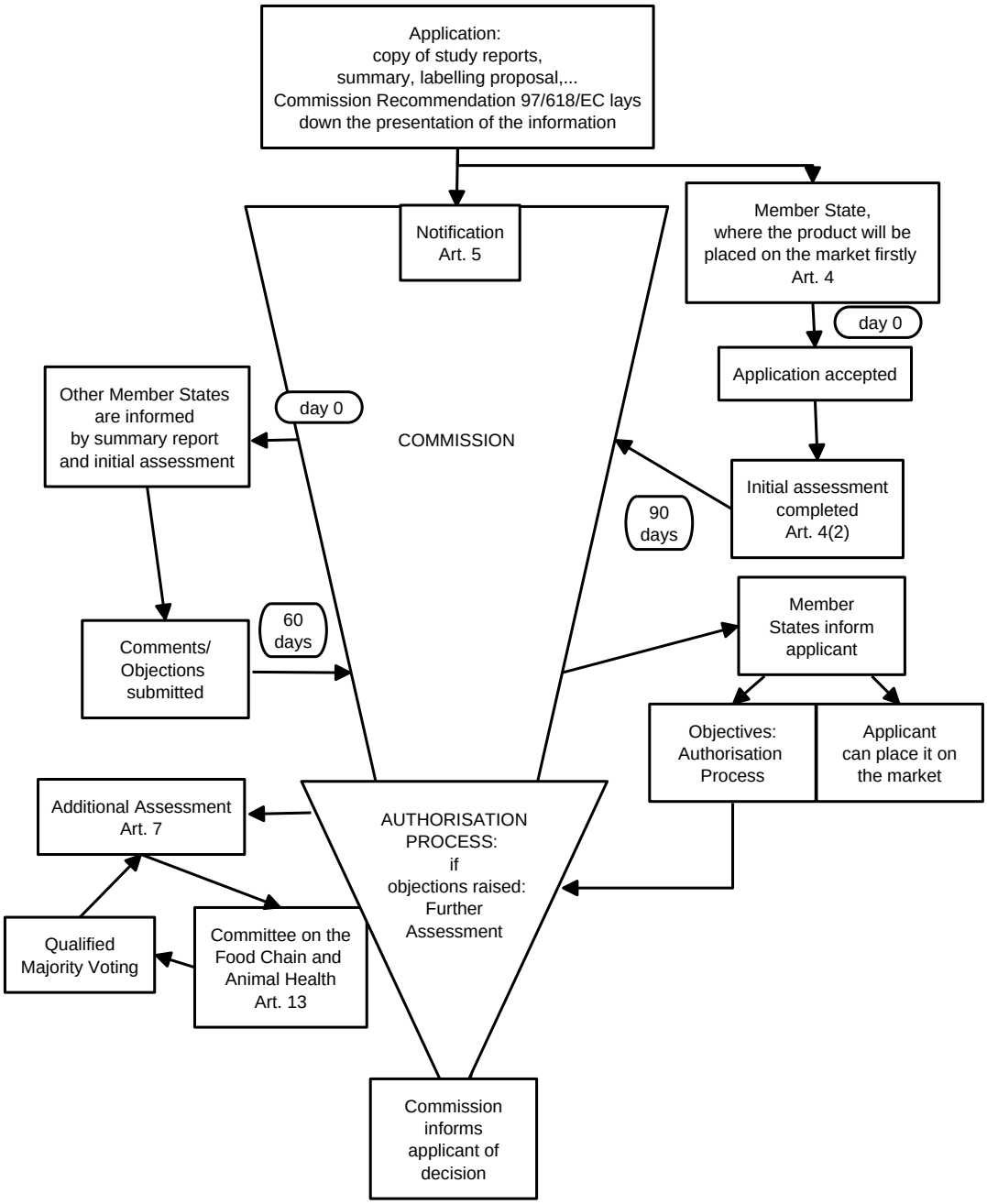

Figure 2: Illustration of the novel food application procedure according the Regulation (EC) No 258/97(adopted by Graham 2000).

Commission Recommendation 97/618/EC

Commission Recommendation 97/618/EC shall be seen as an application dossier for food business operators, which outlines the scientific and safety assessment data required (see Table 1). The scope includes all mentioned categories in Article 1 of the NFR, including GMOs. The *Recommendation* should assist the food business operator in gathering all required information and its correct presentation and layout. Moreover, the focus lies on

presenting the assessment report on substantial equivalence, case-by-case toxicological testing, human consumption patterns and possible adverse reactions (e.g. allergies). Normally, for novel microorganisms, no counterpart exists for establishing substantial equivalence and they are subject to a full assessment. The extended assessment procedure needs to consider containment, possible gut colonization, toxicology, pathogenicity and genetic modification (see Chapter 4.4). However, to simplify the assessment procedure, six different classes of novel food are defined as part of the Recommendation.

For the purpose of this work, two of these six classes are relevant:

Class 2: "**Complex novel food from non-GM sources: including sub-classes"**:

2.1. "The source of the NF has a history of food use in the Community"

2.2. "The source of the NF has no history of food use in the Community"

Class 5: **"GMM and their products, including sub-classes**":

5.1. "The host microorganism used for the genetic modification has a history of use as food or as a source of food in the Community under comparable conditions of preparation and intake"

5.2. "The host microorganism used for the genetic modification has no history of use as food or as a source of food in the Community under comparable conditions of preparation and intake" (European Commission 1997a).

The Recommendation provides a non-exhaustive decision tree to determine which information is necessary to achieve novelty status. Only factors emerged through the novelty are considered (e.g. pathogenicity).

Table 1 illustrates the procedure to determine the necessary information for the assessment of food products or ingredients. Class 2 applies to a "novel non-genetically modified microorganism", with or without history of use. Class 2 is again linked to Article 1(2) d of the NFR. In respect to microorganisms without a history of safe use, regardless of whether they are genetically modified or not, "previous human exposure" is excluded. Considering a microorganism as novel food, attention should focus on safety for human consumption (European Commission 1997a).

Table 1: Identification scheme of essential information for assessment of novel food (European Commission 1997a)

	Commission Recommendation (97/618/EC)		**Novel Food Regulation (258/97/EC) Art. 1(2)**
Novel Microorganisms	Class 2		(d)
	Complex NF from non-GM sources		foods and food ingredients consisting of or isolated from microorganisms, fungi or algae
	2.1. history of food use	2.2. no history of food use	
I.	x	x	Specification (taxonomic unit or chemical compositions)
II.	x	x	Production Process
III.	x	x	History of use of the source of the novel food
IV	*GMOs*		*Effect of the genetic modification on the properties of the host organism*
V.			*Genetic stability*
VI.			*Specificity of expression of novel genetic material*
VII.			*Transfer of genetic material from GMOs*
VIII.			*Ability to survive in and colonize the human gut*
IX.	x	x	Anticipated human intake and its' extent
X.	x		Previous human exposure
XI.	x	x	Nutritional information
XIII.	x	x	Microbiological information
XIII.	x	x	Toxicological information

The Revising of the Novel Food Regulation

Due to several uncertainties, the renewal of the current NFR has been in discussion since 2002 (European Commission 2002b; 2013c). In 2008, a new version was published. The updated version copes with new objectives, such as streamlining the authorisation process by adjusting assessment of traditional food from third countries and improving the efficiency and transparency of the application process. The proposal redefines the scope, defines new technologies and emphasizes legal clarification and consumer empowerment.

As a result, it precisely outlines scope and other important phrases part of Article 3(2): "Novel good", "Traditional food from a third country" and "History of safe use". The proposal extends the scope of novel food by adding "nanotechnology" and food produced by "non-traditional breeding techniques" in short cloning.

In 2011, discussion stopped as Parliament and the Council could not find common ground. An agreement on "cloning" or the use of meat from cloned animals appeared to be the largest obstacle with nanotechnology as another (European Commission 2011a). Consequently, food from cloned organisms remains regulated by the NFR and requires mandatory pre-market approval as long as explicit "cloning legislation" has not been adopted (European Commission 2013b). Following the discrepancies, a new version of the NFR was suggested in 2013 based on agreements accomplished in the previous proposal, but with one main exception. The Commission concluded that it would be better to regulate the "cloning of animals" separately, based on an impact assessment. Therefore, the current proposal is limited to novel food itself. Besides the general principles of Regulation (EC) No 258/1997, the new version focuses on economic ramifications, innovation, simplification and temporal aspects (European Commission 2013c). At present, the original 1997 NFR version is still in force (European Parliament 2011).

The following objectives will be part of the revised 2013 NFR:

- The new proposal will apply to all FBO, no matter which size, with a focus on promoting innovative products.
- If the "history of safe use" applies to a product from a 3rd country and no additional safety concerns arise, it will be seen as safe and therefore imported.
- The simplified and harmonized process will be followed by a reduced and expedited administrative burden for food businesses.
- Under "centralised harmonisation", the proposal will comprise: (1) no simplified application processes by "substantial equivalence" and (2) the responsibility of the initial assessment will be transferred to the Commission and will no longer lie with Member States.
- Data from companies will be treated confidentially for up to 5 years. For this duration of time, no other company is allowed to produce the same product.

- New definitions will clarify scope and the categories of novel food with respect to the General Food Law 178/2002.
- "Nanotechnology" will be included, whereas genetic modification will remain regulated by other Union laws.
- Food, resulting from technologies not used in the Union before, will be considered as novel and further defined.
- Due to a simplified authorisation system, all novel products will be assessed by the EFSA.

The European Parliament and Council must agree to the Commissions' proposal with a focus on obtaining legal status in 2016, at the earliest (European Commission 2008, 2013c).

4.4. Regulation of Genetically Modified Organisms

In the US, the regulation of *genetically modified food* (GM food) and GMOs is limited to necessary safety requirements. Germany, on the other hand, has the reputation of seeing "the risks rather than the opportunities" according Dieter Thomae, Chairman of the Bundestag Committee for Health (Graham 2000). European regulation can be described as being somewhere between both approaches.

European Regulatory Framework

Genetically modified organisms and microorganism used for the production of food, or as food itself, are a unique group of novel food in the European Union and are regulated by national and EU law (Graham 2000).

Since 2004, GMOs have been regulated separately and are no longer part of the NFR unless they fall under another category. Accordingly, the first two categories under Regulation (EC) No 258/97 Article 1(2) were deleted (European Commission 1997b). Similar to food from cloned organisms, GM food remains a topic of debate (van der Meulen and van der Velde 2011).

The European Commission, the Parliament and the Council have been working on a regulatory framework for GMOs and GM food for decades. This has resulted in a long list of Regulations, Directives and Decision. The first explicit regulatory act was implemented in 1990 as Directive 90/220/EEC *on the deliberate release of genetically*

modified organisms into the environment (European Commission 1990b). Due to the need for clarification and amendments, Directive 2001/18 /EC repealed the Directive 90/220/EEC and Regulation (EC) No 1829/2003 *on genetically modified food and feed* (European Commission 2001a; 2003c).

The labelling and traceability requirements are elaborated upon in Regulation (EC) No 1830/2003— to harmonize the European market and trade. Food business operators "from farm to fork" and the consumer himself shall be informed, through written documentation and proper labelling, that the handled food consists of GMOs or contains GMOS (European Commission 2003d). The following scheme shows the regulatory framework below, covering GMOs, GMMs and GM food in a brief abstract:

- **Directive 90/220/EEC** *on the deliberate release of genetically modified organisms into the environment* has been repealed and replaced by (European Commission 1990b):
    - **<u>Directive 2001/18/EEC (GMOs Directive)</u>** *on the deliberate release of genetically modified organisms into the environment, repealing Council Directive 90/220/EEC,* comprises two objectives: Placing GM food for human consumption on the market and releasing GMOs by experimental usages Focuses on:
        - ✓ Procedure for authorising the deliberate release and marketing of GMOs
        - ✓ Methodology for assessing environment risks
        - ✓ EU-wide monitoring procedures (European Commission 2001a).

      The Directive has been amended by:
        - **<u>Regulation 1829/2003 (GMOs Regulation)</u>** *on genetically modified food and feed* focuses on:
        - ✓ Food, feed and ingredients produced from GMOs
        - ✓ Food and feed containing or consisting of GMOs
        - ✓ GMOs for food use
        - ✓ Labelling requirements (elaborated in Regulation 1830/2002) (European Commission 2003c).

- **Commission Regulation 641/2004** *on detailed rules for the implementation of Regulation (EC) No 1829/2003* elaborates the authorisation process (European Commission, 2004b).

- **Decision 2002/623:**

Focuses on environmental risk assessment, on a case-by-case basis, to identify and assess adverse effects of GMOs (additional to Annex II of Directive 2001/18/EC) (European Commission 2002a).

- **Regulation 1830/2003** *concerning the traceability and labelling of genetically modified organisms, the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC* (European Commission 2003d).
    - **Commission Regulation 65/2004** *establishing a system for the development and assignment of unique identifiers for genetically modified organisms* describes the implementation of the traceability system for GMOs introduced to market, laid down in Regulation 1830/2002. Each product receives a unique identifier (nine-digit database code) (European Commission 2004a).
- **Directive 90/219/EEC** *on the contained use of genetically modified microorganisms* has been amended by (European Commission 1990a):
    - **Directive 2009/41** *on the contained use of genetically modified microorganisms* (GMM Directive) focuses on GMMs for experimental usage under research and industry, whereas it does not include the release of GMMs (European Commission 2009a).
- For the release of GMM, or the placement of GMM consisting or containing products on the market, the requirements in Regulation (EC) No 1829/2003, together with Directive 2001/18/EC, apply (European Commission 2001a; 2003b).
- **Regulation (EC) No 1946/2003** *on transboundary movements of genetically modified organisms* lays down the implementation of the Cartagena Protocol (European Commission 2003b).

Additionally, guidelines are helpful tools for FBO to cover the full risk assessment of GMMs, as the *Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed* (GMM Guidance) by the EFSA.

Contained Use of Genetically Modified Microorganism

Especially for GMM, Directive 2009/41 applies. The definition of a GMM is laid down in Article 2(b):

> "genetically modified micro-organism' (GMM) means a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination; within the terms of this definition"
>
> – (European Commission 2009a).

Techniques through which a GMM might occur are listed in Annex I, Part A, but should be viewed as examples, since other techniques could also lead to genetic modification (Bar-Yam et al. 2012). "Contained use" refers to all activities where release and contact with the environment and population is studiously avoided, meaning research facilities and industrial test plants. The Directive excludes mechanisms that are not considered as recombination of DNA, for example in vitro fertilisation, self-cloning, natural processes as conjugation, transduction, mutagenesis and cell fusion, including protoplast fusion (Annex II, Part B) (European Commission 2009a). Market release and placement in compliance with the GMOs Directive and Regulation is also excluded (European Commission 2001a; 2003c).

The responsible person shall carry out a comprehensive assessment of potentially adverse risks, including identification of harmful effects, their likelihood, severity and GMM activity characterisation. The assessment procedure itself is divided into three steps: identification of harmful properties of the GMM, definition of the risk level (classes defined in Article 4(3)) and establishment of sufficient containment measures.

Working with GMM in contained conditions requires a preceding notification submitted by the user to the competent national authority. The NCA approves the assessment for completeness and correctness of the risk assessment and may ask for further information, if relevant (see page 30). In the case of an unwanted release of GMMs into the environment, the NCA shall ensure that a suitable emergency plan is established and all involved parties are opportunely informed (European Commission 2009a).

Deliberate Release into the Environment and Genetically Modified Food and Feed

Food, including GMOs or GMMs, is more stringently regulated than conventional novel food. Its' authorisation requires a comprehensive case-by-case safety assessment before it can be placed on the market. The two authorisation steps aim to protect the environment, human health and consumer interests. Due to ongoing amendments, Directive 2001/18/EEC and Regulation (EC) No 1829/2003 are similar and overlapping in several points.

Directive 2001/18/EEC covers the deliberate release of a GMO, GMM or a combination of GMOs where no specific containment measures are maintained into the environment. The GMOs Directive is divided into two parts, Part B and Part C. However, activities under both parts require NCA notification.

Part B only covers the release of GMOs for controlled or experimental purposes. The notification in this case must include a technical dossier and an ongoing environmental risk assessment, stipulated in Annex II, Section D. The risk assessment shall identify and evaluate, on a case by case basis, indirect and direct effects on the environment and human health by the GMO, with a focus on developing a risk management strategy, if necessary. The NCA has 90 days to acknowledge the notification or to reject it. The given authorisation is only valid in the Member State where the notification has been applied. In contrast, Part C covers the commercial release of GMOs to any third party. The notification procedure is similar to Part B, but requires the involvement of the Commission and other Member States (see notification procedure Regulation (EC) No 1929/2003). The final decision is consequently adopted by comitology and valid up to ten years (GMOs Directive, Article 18) (European Commission 2001a). In the case of a rejection, the company or notifier has the possibility to submit the same notification to another Member State and see whether it decides in its favour (EFSA 2009a).

Three steps are mandatory to obtain a Part C authorisation. First, an environmental risk assessment (Annex II, Section D), second, a monitoring and reporting plan and, third, a notification with detailed information about the GMO, its handling and labelling. Exemptions from the GMOs Directive are listed in Annex IA, Part 2 and Annex IB, which are similar to the excluded techniques listed in the GMM Directive (European Commission 2001a). Further details and information regarding the content of the

notification and the authorisation process are elaborated below, referring to Regulation (EC) No 1829/2003.

In terms of food, Regulation (EC) No 1829/2003 has been given higher priority, although it only covers food and feed produced "from" GMO and not "with" GMO. Processing aids produced with the help of GMM and fulfilling a technical purpose are not covered by this Regulation. The determining criterion is whether or not genetically modified DNA is part of the product.

The scope includes pre-market approvals of:

- "food and feed containing or consisting of GMOs*"* (e.g.cornstarch)
- "food and feed produced from or containing ingredients produced from GMOs" *(*polenta produced of cornstarch*)*
- "GMOs for food and feed use (e.g. corn, GMM)" (definition in Article 2(8)) (European Commission 2003c).

The whole authorisation process follows the "one door one key" principle, as the granted authorisation of a GM food is legally binding among all European Member States. Regulation (EC) No 1829/2001 simplifies the process by combining the requirements of Directive 2001/18/EC with the requirements for implementing food or feed on the market. Subsequently, the responsible person does not need to separately apply for the GMO itself nor its use in food or feed. The organism is allowed to be released into the environment.

The person responsible for the application is the one who uses the GMO as a source material, or the person who intends to place the food on the market. The application is assessed by the EFSA with the Community and Member States as risk management bodies (European Commission 2001a; 2003c; van der Meulen and van der Velde 2011). It must be emphasized that, according to the law, all GMOs are prohibited unless they have been approved. For better understanding the authorisation procedure according to Regulation (EC) No 1829/2001 is elaborated upon in three parts and illustrated below in Figure 3.

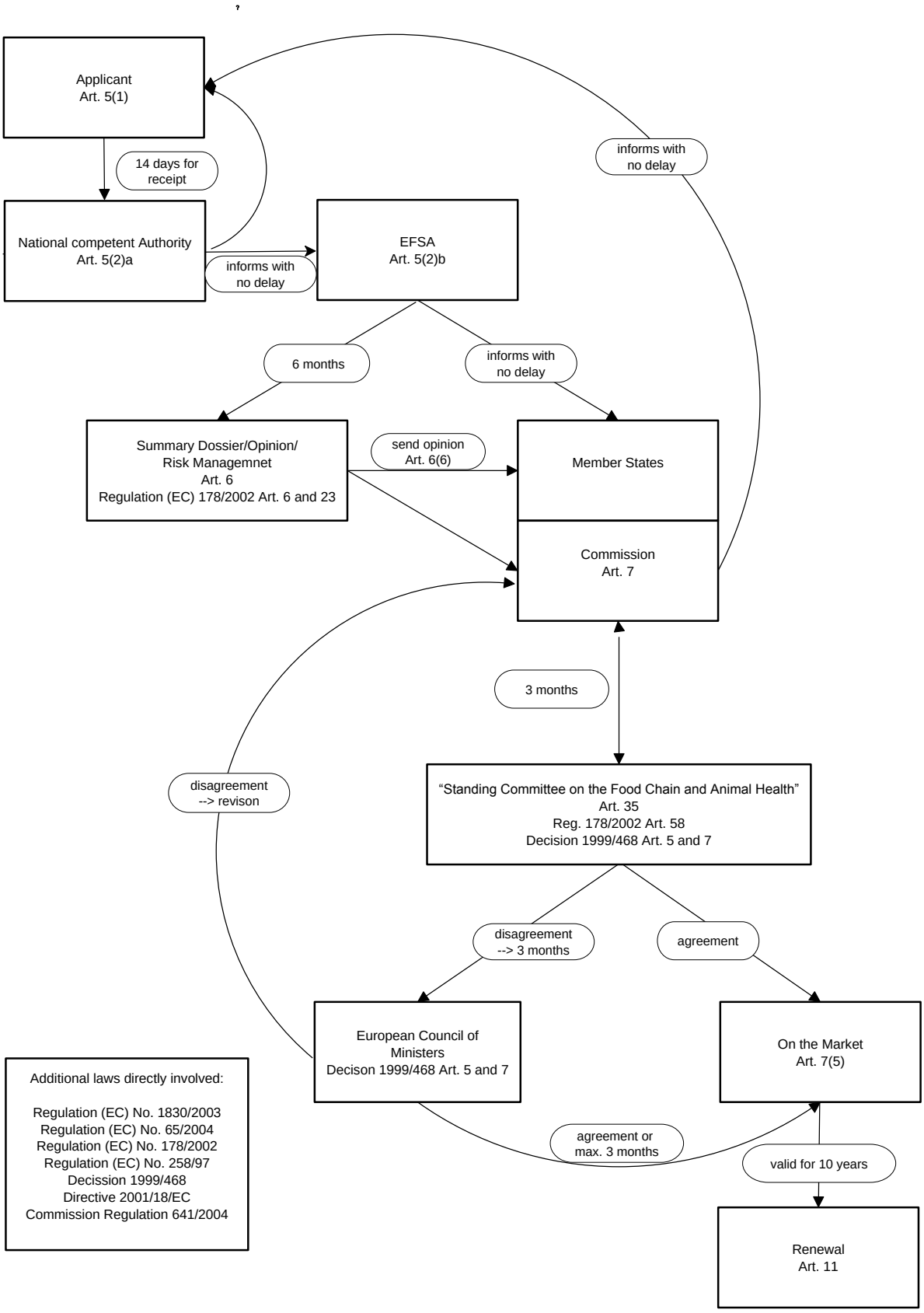

Figure 3: Authorisation procedure for GM food (according to Regulation (EC) No 1829/2003)

Phase 1: Submitting an Application

The authorisation process starts with a notification to the respective national food safety authority, where the product will firstly be placed on the market. The NCA immediately forwards the information to the EFSA. The EFSA hereafter informs the other Member States and the Commission about the application. The NCA approves the notification for compliance and informs the applicant of receipt within 14 days. In contrast to the NFR, the responsibility and impact of the national authorities is limited.

The applicant can define which information should be treated confidentially according to Article 30, excluding information concerning the GMO and product portrayal. Under Commission Regulation (EC) No 641/2004 the applicant must submit a labelling proposal and plans for monitoring, detection and sampling. Additionally, Article 5(3) of Regulation (EC) No 1829/2003 lays down the mandatory information necessary to prove safety and suitability of the GM food. The application shall at least include: peer-reviewed studies, comparative data to conventional food, descriptions of production and manufacturing, exclusions of ethical concerns, methods for detection and sampling, and samples of the food itself. Food containing GMOs or consisting of GMOs shall be additionally accompanied by a technical dossier and environmental monitoring plan as required by Annex III, IV and VII of Directive 2001/18/EC (European Commission 2003c).

Phase 2: Safety Assessment

If the application is complete and favourable, EFSA has 6 months for assessment with an opportunity to prolong the process if further information is required and requested from the applicant. EFSA is responsible for the environment, human and animal health safety assessment, but can ask NCAs to carry out the respective tasks. If required by a product, EFSA may request Member States to adopt an environmental risk assessment in accordance with Article 4 of Directive 2001/18. The Commission's technical advisory unit, the "Community Reference Laboratory", is responsible for the validation of all applied detection and identification methods proposed by the applicant, in accordance with Article 6(3)[d].

All gathered information is published as a final opinion by the EFSA, including a generic safety assessment and the following data:

- Name and address of the applicant
- Designation of the food
- Information referred to the Cartagena Protocol, if required
- Proposal for product labelling
- Monitoring plan
- Validation method for detection and identification
- Post-market monitoring plan and protection measures
- Detection and identification methods validated by the "Community Reference Laboratory"
- Environmental monitoring plan for GM plants with regards to Directive 2001/18/EC (Regulation (EC) No 1829/2003 Article 6).

The public is allowed to comment within 30 days (European Commission 2003c).

Phase 3: Final Decision (Comitology Procedure)

The final step, the official decision finding, comprises the same process used in all legislative decision-making procedures (Council of the European Union 1999). Within three months of receiving the EFSA's final opinion, the Commission publishes a proposal which can be granted or refused. The *Standing Committee on the Food Chain and Animal Health* represents the Member States, and eventually decides if the Decision shall be adopted. Alternatively, if the Committee does not agree with the proposal, it is the burden of the European Council of Ministers to vote. The Council of Ministers has 90 days to reach a qualified majority. In case of a rejection, the Commission must rework the proposal. If the Council of Ministers is not able to reach consensus on the proposal in time, the proposal is returned to the Commission and finally adopted. The authorisation is addressed to the applicant and granted as a monopoly for up to 10 years within in the Community. For a subsequent 10 years, renewal of a further notification and authorisation process is required. Within these 10 years, the authorisation is impeachable (European Commission 2003c).

Similar is the procedure for subjects needing authorisation in accordance to Directive 2001/18 for an environmental impact assessment. In comparison to Regulation

1829/2003, the national authorities are responsible for the environmental risk assessment of cultivars by preparing a report within 90 days. The EFSA is only asked for additional advice in case of disagreements among Member States (European Commission 2001a; 2003c).

Labelling

Labelling is required for all products which contain, consist or are manufactured from GMOs and GMMs in an amount higher than 0.9%, without exemption (European Commission 2001a; 2003c; 2003d). The established threshold is necessary for preventing the FBO against unavoidable GMO traces introduced into the product through any ingredients and production steps. Article 13 of Regulation (EC) No 1839/2003 stipulates the concrete wording obligatorily displayed on the product, which can be either "genetically modified", "produced from genetically modified", or "produced from [name of the ingredient]" (European Commission 2003c). Supplementary requirements, laid down in Regulation (EC) No 1830/2003 concerning the traceability and labelling of GMOs, as well the general labelling rules provided for food and feed (Directive 2000/13/EC and Regulation (EC) No 767/2009) must be considered (European Commission 2000a; 2003d).

Which Legislation Applies and When?

The applicant can decide if he/she complies with Directive 2001/18/EEC, Regulation (EC) No 1829/2001, or both according to priority and product. For food and feed containing or consisting of GMOs, the applicant can choose between the "one door, one key" principle under Regulation (EC) No 1829/2003 in order to obtain a single authorisation for use in food and feed (GMOs Regulation), or deliberate release into the environment (GMOs Directive) in one process or a separate procedure. The other possibility would be to comply with the legislation separately, covering the environmental release by the GMOs Directive and the use in food or feed by the GMOs Regulation (European Commission 2001a; 2003c).

A producer of GMMs used for food or food products (e.g. enzymes) can decide whether to submit the application only under the GMOs Regulation or under the GMOs Regulation and the GMOs Directive. In terms of a contained use, only the GMMs Directive applies. If another producer, for example, adds this GMM to a food with the

intent of placing it on the market, the authorisation must comply with the GMOs Regulation (Cana and Schliessner 2005/06).

The following examples and Figure 4 illustrate the regulatory concept:

- GMMs and GMOs for food and feed without cultivation (e.g. GMM is not viable), require a single application for food and feed purposes. For products that are intentionally used as feed and food, authorisation for both purposes shall be applied, or not at all (Regulation (EC) No 1829/2003).
- For GMMs and GMOS for food and feed with cultivation (e.g. GMM is viable), an application for both cultivation and food/feed purposes is required (Regulation (EC) No 1829/2003 and/or Directive 2001/18/EC).
- If the GMO or GMM is not used as food or feed, an authorisation for cultivation is sufficient (Directive 2001/18/E C) (European Commission 2001a; 2003c).

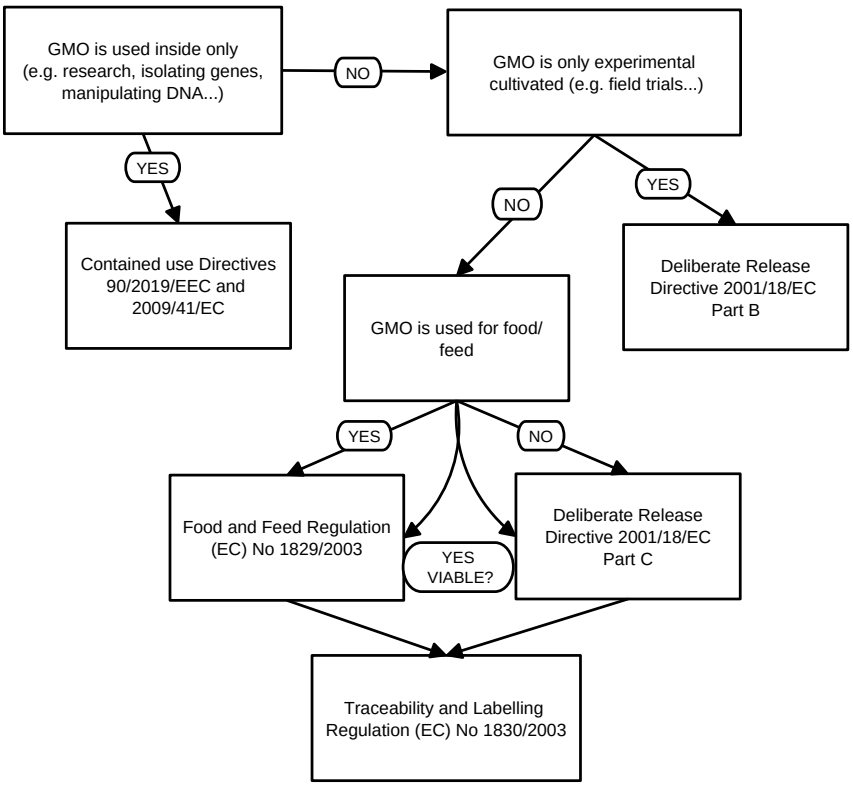

Figure 4: Simplified structure of the EU GMO regulatory framework

The first step is the development of the GMM in the laboratory; therefore, Directive 90/219/EEC and 2009/41 applies. If the GMO is suitable it must be tested outside contained use, which is covered by the Deliberate Release Directive 2001/18/EC. For approved release, a safety assessment must be carried out, where the burden of proof falls on the applicant. To bring the product to market permission under Regulation (EC) no. 1829/3002 must be granted, or in combination with Directive 2001/18/EC, when required. The GMO authorisation process is often subject to fierce criticism, as it is time consuming, cost-intensive and complicated (European Commission 2011b).

Table 2: Timeline for authorisation of GM food (van der Meulen and van der Velde 2001)

Time	*Step*
14 days	Member States notify EFSA and send receipt to applicant
6 months	Risk assessment by EFSA
Not defined	If additional information are required
3 months	Draft proposal by Commission
Not defined	Committee opinion
3 months	Council if necessary
Not defined	Commission adopts Decision
>54 weeks	In total

Table 2 illustrates the approximate duration of the procedure, which can be prolonged for years. In many cases the Committee or the Council fails to reach a significant majority, followed by delayed authorisation and finally adoption by the Commission (European Commission 2003c; van der Meulen and van der Velde 2001).

To date, 41 GMOs for either food or feed are registered and authorised in the EU. Of those, no food product containing GMMs or derived from GMMs is on the market. GMMs intended for the use of feed as bacterial and yeast biomass are currently on the waiting list for renewal (European Commission n.d.a). The situation is unlike GMM-produced enzymes, which have been on the market for decades (Aguilera et al. 2013).

Regulation in the United States

The USA has a less stringent approach of regulating GMOs, since no threshold exists and no specific labelling is required under law. The safety assessment of GM food and GMOs is principally based on "substantial equivalence" to existing conventional products. If

equivalence is approved, the product is regulated in the same way as conventional food or microorganisms and handled as "Generally Recognized as Safe" (see Chapter 4.1.).

The *Coordinated Framework of Biotechnology* is the main approach for regulating biotech food in the USA (Cochran 2003). Three US authorities are involved through pre-existing regulations (e.g. FD&C Act): the *US Department of Agriculture* (USDA), the *Food and Drug Administration* (FDA) and the *Environmental Protection Agency* (EPA). These three agencies deal with the commercial distribution of GMMs, plants, food and drugs, unlike the *National Institute of Health Guidelines for Research Involving Recombinant Synthetic Nucleic Molecules* (NIH Guidelines) (National Institutes of Health 2003; United States Department of Agriculture/APHIS n.d.).

The NIH Guidelines are the most widely used guidelines for defining safety practices and containment measures for clinical research using recombinant DNA. These guidelines are only obligatory for research institutions who receive federal grants for respective research (National Institutes of Health 2003).

The responsibilities of the involved institutions are as follows:

- The USDA, in particular the *Animal Health and Inspection Service* (APHIS), regulates the first step of releasing GMOs into the environment through distribution, field tests, interstate movement and release (plants, insects and microorganisms) that may be harmful to the environment and animals. Introduction of a GM plant requires an APHIS notification or a permit, depending on the risk level (United States Department of Agriculture/APHIS n.d.).
- If the company wants to implement the GMO as food and no GRAS status has been granted, the FDA must be notified. The FDA is responsible for genetically modified derived medical products and any GM food and feed. Only if the GM food does not gain GRAS status will pre-market approval be required, as for food additives (Bar-Yam et al. 2012; Cochran 2012).
- The EPA regulates newly developed microorganisms for commercial use under the *Toxic Substances Act*. Bio-pesticides, including Bt toxins, are regulated under the *Federal Insecticide, Fungicide and Rodenticide Act*. Similar to the EU environmental safety assessment, anyone who produces and imports microorganisms is obliged to

submit a *Microbial Commercial Activity Notice* (MCAN) or a *TSCA Experimental Release Application* (TERA), depending on the intended application (Bar-Yam et al. 2012; U.S. Environmental Protection Agency 2012).

If a product is free of non-GRAS ingredients and is not covered by the EPA, the applicant has two possibilities to bring it to market: either to introduce it immediately or to consult the FDA voluntarily. However, consulting the FDA involves a detailed description and characterisation of the GMO by the company (Bar-Yam et al. 2012; Cochran 2012).

4.5. Conclusion of Chapter 4

In comparison to the USA, the EU has only recently started coping with the regulation of bacteria. Although the US and European regulatory approaches concerning microorganisms are often influenced by interests, internal trade security and politics, some points are similar. The QPS system is based on the US GRAS approach and shows some similarities (e.g. "history of safe use") (see
). However, the GMOs regulatory framework in Europe is one of the strictest worldwide and, in terms of safety assessment, thresholds and labelling is not comparable with US regulation. Under European law, it is no longer possible to classify a GMO as a "substantial equivalent" to a conventional product, as is still common in the USA.

To ensure the highest level of safety the "precautionary principle", as laid down in Article 7 of the GFL, functions as a last resort within the Union. Nevertheless, food law harmonization within Member States remains problematic, as Directives still represent a huge part of the regulatory framework. The interpretation of food law varies by Member State, depending on pre-existing national laws. Most US regulatory frameworks dealing with microorganisms focus on the properties of the product or microorganism and not on the production process itself. In contrast, the EU aims to concentrate on these objectives. The US simplifies the application process for novel substances and GMOs by applying the same rules as for conventional food. Furthermore, the FDA delegates the burden of proof towards companies. There is no regulatory distinction between food and feed and a specific novel food legislation does not exist. All these conveniences facilitate the introduction of innovative products to the US market.

Within the EU, the whole application process, for example novel food and GMOs, is challenging and expensive. Additionally, it has not been specified from which state of science and knowledge, "novel food" is not novel anymore and can be regulated as conventional food. Table 3 and Table 4 summarize the differences and similarities of legislation discussed in Chapter 4 .

Table 3: EU and US way to regulate GM topics of concern

	US GMOs	*EU GMOs*	*International GMOs*
Transfer of genes	EPA and APHIS	Directive 2001/18/EC	Cartagena Protocol on Biosafety
Effects on environment and other species	EPA and APHIS	Directive 2001/18/EC	Cartagena Protocol on Biosafety
Human/Animal health	EPA and FDA	Regulation 1829/2003	
Health of laboratory workers	NIH Guidelines	Directive 2009/41/EC	
Accidental release of GM strains	NIH Guidelines	Directive 2009/41/EC	

Table 4: The differences and similarities of the in Chapter 4 mentioned regulatory approaches

	EU NFR	*US GRAS*	*EU QPS*
Age	Since 1997	Since 1958	Since 2007
Legal Status	Regulation (EC) No 258/97	FD&C Act, CFR 21	No legal status (only in Reg. 562/212)
Scope	All Substances	All Substances	Biological agents and their products
Burden of Proof	NCA/Notifier	Notifier	EFSA
Focus	Novelty/"Substantial Equivalence"/ History of Safe Use	Intended Use/Product/ "Substantial Equivalence"/ History of Safe Use	History of safe use/ Microorganism/ AMR
List	Open-List/ "Grandfather-List"	Open-List/ "Grandfather-List"	Positive List
List-Update	No regular update	No regular update	Annual update

5. Safety Assessment of *Lactobacillus reuteri*, Strain DSM 17983

5.1. Identity of the Organism

This chapter discusses the characteristics of *Lactobacillus reuteri* strain ATCC 55738, with a focus on the modified daughter strain DSM 17938. The focus strain is identified as DSM 17938 (deposited by the *Deutsche Sammlung von Mikroorganismen und Zellkulturen*) is commercially named *L. reuteri* Protectis® by BioGaia (AG Stockholm, Sweden). It works as an ingredient in several probiotic products. The original strain, ATCC 55738, was intensively researched and deposited in the American Type Culture Collection (ATCC). It is also known as SD2112, ING and MM53 (hereafter *L. reuteri* ATCC 55730) (J Heimbach LLC 2008). Supplemented products containing *L. reuteri* ATCC 55730 have been on the market since the early 90s, whereas those with *L. reuteri* DSM 17939 have been available since 2007 (Casas and Dobrogosz 2000; Connelly 2004; J Heimbach LLC 2008).

Description

Due to the heterogeneous properties within the strains, the taxonomic characterisation can be challenging and should be based on genotypic (DNA based composition, 16sRNA gene sequence similarities, DNA-DNA hybridization,), phenotypic (cell morphology, motility, fermentation pattern, antibiotic susceptibility, etc.), as well as chemotaxonomic (peptidoglycan) and ecological studies (habitat, source, etc.) (Mattarelli et al. 2014). According the EFSA *Panel on Dietetic Products, Nutrition and Allergies* (NDA panel), the following methods should be used for the characterisation of bacteria which are potential candidates for health claims, as *L. reuteri* strain DSM 17938:

– "**Species identification** by DNA-DNA hybridisation or 16S rRNA gene sequence analysis"

– "**Strain identification** by DNA macro-restriction followed by *pulsed-field gel electrophoresis* (PFGE), *randomly amplified polymorphic DNA analysis* (RAPD), or other internationally accepted genetic typing molecular methods" (EFSA 2009c).

The important and well described bacterial genus *Lactobacillus* is part of a phylogenetically heterogeneous group of gram-positive, non-spore-forming, normally non-motile, catalase negative (pseudocatalase activity has been observed) lactic acid bacteria, shaped as rods or coccobacilli. Species of *Lactobacillus* produce lactic acid as a product of carbohydrate homo- or hetero- fermentation and are categorised as microaerophilic, as well as chemo-organotrophic (Felis and Dellaglio 2007). The base composition of guanine and cytosine (GC) is normally low, between 32-53 mol% (Adams and Moss 1995; Felis and Dellaglio 2007; Salvetti et al. 2012). Optimal growing temperatures lie between 30 and 40°C (with possible growth between 2 to 53°C) and the favourable pH ranges between 3 and 8, the optimum being 5.5 to 6.2. Species of *Lactobacillus* are ubiquitous, as they prefer the carbohydrate rich conditions given in food, environment and the human and animal gastrointestinal tract. The strain has a long history of use in food production (Mattarelli et al. 2014; Tannock 2004).

L. reuteri is the only *Lactobacillus* strain that inhabits the complete intestinal tract of human as well as of animals. It has also been isolated from human breast milk (e.g. strain ATCC 55730) (Casas and Dobrogosz 2000; Sinkiewicz and Ljunggren 2008; Rosander et al. 2008). As its own phylogenic group, *L. reuteri* belongs to the obligate heterofermatives and therefore produces lactate as well as ethanol/acetate and carbon dioxide in equimolar amounts (Adams and Moss 1995; Salvetti et al. 2012). The GC content usually ranges from 40-42 mol% (strain DSM 17938 shows a GC content of 38.59 mol% and strain ATCC 55730 39.3 mol%) (Årsköld et al. 2008; Båth et al. 2005; O'Sullivan 2008). The type of lactic acid produced is characteristic of a species and does not vary within (Connolly et al. 2005). The reuteri species produces a DL lactic-acid isomere and contains peptidoglycan from type Lysin-D-Asparagin, whereas strain ATCC 55730 preferentially generates L-lactic acid (at a ratio of 3:1) (Årsköld et al. 2008; Saulnier et al. 2011). *L. reuteri* ATCC 55730 seems to possess genes for the Embden-Meyerhof pathway as well the phosphoketolase pathway to ferment glucose or pentose (Årsköld et al. 2008; Felis and Dellaglio 2007). All *L. reuteri* strains are capable of metabolizing the antimicrobial compound reuterin (3-hydroxypropionaldehyde) as an intermediate product in a two-step conversion process of Glycerol to 1,3-propanediol, yielding NAD^+ from NADH. Vitamin B12 serves as a coenzyme (Santos et al. 2011). Reuterin production has been shown to be stimulated by interactions with other bacteria

and may, under certain circumstances, create a selective growth advantage for the *Lactobacillus* strain (Chung et al. 1989). The excreted broad-spectrum antimicrobial substance has been reported to work against viruses, fungi and bacteria, but can be also be converted to the toxic compound acrolein (Schaefer et al. 2010; Talarico et al. 1988). Furthermore, resistance properties against intestinal bile acids are described. On the one hand, it is assumed that bile acid limits the growth of strain ATCC 55730 (Morelli et al. 2012; Saulnier et al. 2011). On the other hand, strain ATCC 55730 is capable of surviving one hour at a pH of 2.7., leading to physiological advantages as a probiotic.

At a low pH the strain synthesizes phosphatidylglycerol, cardiolipin, acidic phospholipids and upregulates other genes (e.g. ATPase with chaperone activity, putative esterase). Therefore, it can survive the harsh conditions found in the upper gastrointestinal tract (Wall et al. 2007). However, in vitro testing of different *L. reuteri* strains showed that the survival rate of strain DSM 17938 in gastric acid is not as good as other strains. The adhesion capacity varied greatly within the *L. reuteri* strains. According to the results of *L. reuteri* strain DSM 20016, it seems to carry the best adhesion properties (to Caco-2, HT29 and LS174T cell lines), whereas strain DSM 17938 showed less than average results (Jensen et al. 2012). These differences can be explained by the strain specific genetic variations of mucus binding protein (MUB) or MUB-like protein genes located in plasmids (MacKenzie et al. 2010). Considering the impact on gut barrier function, strain DSM 20016 and 17938 are the only ones among those studied which could increase transepithelial electrical resistances (Jensen et al. 2012). Furthermore, strain ATCC 55730 comprises complete pathways for vitamin B12, folate and threonine synthesis and possibly thiamine synthesis. Although the strain is more susceptible to bile salts, there is evidence that it harbours competitive genetic advantages indidcated by its' ability to survive in breast milk by utilizing lactose, galactose and galacto-oligosaccharides (Saulnier et al. 2011).

Classification and Taxonomy

From a taxonomic point of view, the genus *Lactobacillus* is thoroughly described and supported by phylogenetic molecular taxonomy and 16S rRNA gene sequence methods (EFSA 2007). Originally, the taxonomic classification of Lactobacillus had rested on phenotypic and fermentative properties, followed by a grouping in three classes (obligatory homofermentative, facultatively heterofermentative, obligatory

heterofermentative) (Hammes and Vogel 1995; Klein et el. 1998). The first phylogenetic analysis of the genus was performed by Collins et al. (1991) and represents the starting point of a constantly growing number of novel species over the years. According *Bergely's Systematic Outline of the Prokaryotes* the genus *Lactobacillus* belongs to the phylum Firmicutes. Further taxonomical classification is listed in Table 5 and 6 below. *Pediococcu*s and *Paralactobacillus* remain the closest relatives of *Lactobacillu*s within the same family (Garrity et al. 2004; Salvetti et al. 2012). However, *Paralactobacillu*s has recently been integrated to the *Lactobacillus* genus due to undistinguishable characteristics determined through multilocus sequence analysis of 16sRNA (Haakensen et al. 2011). The reclassification still remains a topic of discussion (Mattarelli et al. 2014). The closest phylogenetic relative is the family *Leuconostocaceae* (Felis and Dellaglio 2007). At firsrt, *L. reuteri* was incorrectly designated as the strain *L. fermentum* IIb. Although *L. reuteri* and *L. fermentum* demonstrate phenotypical similarities, they differ in molecular phenotypology, growth properties, antibiotic resistances (tetracycline) and peptidoglycan types (Egervärn et al. 2009; Klein et el. 1998). The species *L. reuteri* was first described as an independent strain by Kandler et al. (1980) according to DNA–DNA homology. In 1982, the results were published in the *International Journal of Systematic Bacteriology* (LPSN n.d.). To date (April 2014), the genus includes 201 specified species with a great variety of properties. The last species added was named as *L. apis* (Killer et al. 2014). Due to the steady growing number of new species, some authors preferred to split the Lactobacillus species into smaller groups, according to their phenotypic data. For instance, Salvetti et al. (2012) and Felis and Dellaglio (2007) added 15 subgeneric species to the *L. reuteri* group: *L. alvi, L. antri, L. coleohominis, L. fermentum, L. frumenti, L. equigenerosi, L. gastricus, L. ingluvei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. secaliphilus, L. vaginalis.* All of these species are obligate heterofermenters, except *L. anis* and *L. secaiphilus*.

Figure 5 illustrates the evolutionary relationship between the different lactobacillus species in the form of a phylogenetic tree. Genome sequencing is an important tool for grouping bacteria, as it provides information on their evolutionary background and compares data amongst related species (Felis and Dellaglio 2007).

Rosander et al. (2008) characterised strain ATCC 55730 and DSM 17938 phenotypically and genotypically. According to the *National Center for Biotechnology*

Information (NCBI), the genome of strain ATTC 55730 is completely determined. The 16S rRNA gene sequence of strain ATTC 55730 is deposited under "Accession number" EU394679 at "Genbank" (NCBI n.d.a; Saunier et al. 2011).

A comprehensive characterization of 126 gene encoding putative extracellular proteins of *L. reuteri* ATCC 55730 concluded that most of the proteins are common in gram-positive bacteria, whereas 24 are restricted to *L. reuteri*. The number of excreted enzymes is higher than in related species (44 genes), whereas encoded proteins for ABC transporters are lower (13 genes) (Båth et al. 2005). In the case of strain DSM 17938, a total of 2299 potential genes were analysed for safety reasons and compared with complete genome sequences of other *L. reuteri* strains (F275 and 100-23). The results showed a compliance with more than 98% of the genes and no unusual properties (O'Sullivan 2008, p.109-118). Three different studies agree that *L. reuteri* strains can be clustered into two clades. Strains ATCC 55730 and its daughter strain DSM 17938 comprise one phylogenetic group, whereas strains DSM 20016, mm4-1a, fj1, 6475 and 4659 comprise another (Jensen et al. 2011; Oh et al. 2010; Saulnier et al. 2011). The investigation conducted by Saulnier et al. (2011) of two *L. reuteri* strains (ATTC 55730 and ATCC PTA 6475) showed that even related strains of the same species share only 70% of their genes. This stresses the importance of comprehensive differentiation among the same species.

Table 5: Taxonomical classification of Lactobacillus reuteri strains ATCC 55739 and DSM 17938

PHYLUM	FIRMICUTES
CLASS	Bacilli
ORDER	Lactobacillales
FAMILY	*Lactobacillaceae*
GENUS I	*Lactobacillus*
SPECIES	*Lactobacillus reuteri*
STRAIN	*ATCC 55730*
	DSM 17938
GENUS II	*Paralactobacillus*
GENUS III	*Pediococcus*

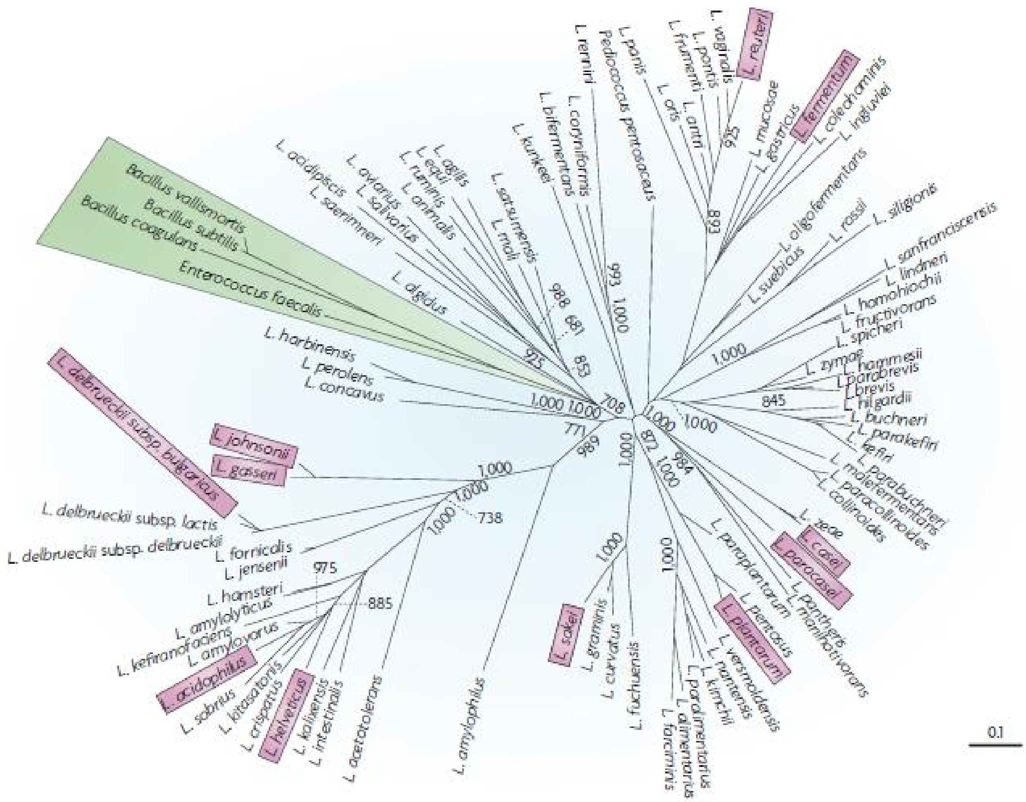

Figure 5: Phylogenetic tree based on 16rRNA gene sequence, displaying the evolutionary relationship between Lactobacillus species. *Species which are shaded in pink represent the determined genome sequence. Species shaded in grey are outgroups (adopted by Ventura et al. 2009).*

Table 6: Taxonomic classification of Lactobacillus reuteri according to Bergey's Systematic Outline of the Prokaryotes

	L. reuteri **ATCC 55730**	***L. reuteri*** **DSM 17938**
GC (mol%)	39.3% (O'Sullivan 2008, p.109-118)	38,59 (Årsköld et al. 2008)
Growth Temperature	37°C	
Prefered Agar	MRS broth/agar (Rosander et al. 2008)	
	Anaerobic growth (Rosander et al. 2008)	
Fermentation pattern	Positive for: L-arabinose, ribose, galactose, glucose, maltose, lactose, melibiose, saccharose, rafinose, gluconate (Rosander et al. 2008)	
Colony Colour	Cream (Singh et al. 2012)	

Characterisation of Strain DSM 17938 and ATCC 55730

Although *L. reuteri* strain ATCC 55730 has been popular for its probiotic benefits, it is also known for its intrinsic antibiotic resistance genes (Casas and Dobrogosz 2000; Connolly 2004; Egervärn et al. 2009a;b; Klare et al. 2007; Klein et al. 2000). Nevertheless, non-transmissible intrinsic features are considered safe according to EFSA, even though gene transfer can never be excluded (EFSA 2005b; Wilcks and van Hoeck 2012). It appears beneficial to not take the risk of removing the potential resistance genes by plasmid-curing techniques (Rosander et al. 2008; Wilcks and van Hoeck 2012).

Table 7: Summary of harboured resistances of Lactobacillus reuteri strain ATCC 55730

***L.reuteri* strain ATCC 55730**	**MIC**	**MIC Breakpoint***	**Gene location**	**Resistance genes**
Tertracycline (Egervärn et al. 2009; Kastner et al. 2006)	>256	>16	Plasmid	tet(W)**
Lincomycin (Kastner et al. 2006; Rosander et al. 2008)	>16	1	Plasmid	lnu(A) but sensitive to clindamycin
Clindamycin (Rosander et al. 2008)***	<0.125*	1	No genes involved	
Vancomycin vanA/B/C (Klein et al. 2000)	>64	Not required	No genes involved	
Gentamicin, ciprofloxacin, trimethoprim, cefotaxime, ß-Lactams… (Klein et al. 2000; Kastner et al. 2000)	Phenotypic resistances****		No genes involved (Intrinsic Structures)	

* For *L. reuteri* values above the specified amount require further investigations (EFSA 2008b)

** Mediates ribosomal protection against tetracycline (Kastner et al. 2006)

*** Phenotypic resistance to clindamycin (Kastner et al. 2006)

**** By Disc Diffusion test

Egervärn et al. (2009b) investigated 32 different *L. reuteri* strains of resistance genes. In 28 strains, atypical MIC levels (> 64 µg/mL) for tetracycline were observed. In 24 of these 28 strains, the resistance was located on the tet(W) gene. *L. reuteri* strain ATCC 55730 did not test positive for erythromycin resistance (MIC > 256 µg/mL). Among others, the tet(W) gene has commonly been found in gut related bacteria in different populations worldwide which can be attributed to conjugation via transposons (De Vries

et al. 2011; Egervärn et al. 2009b; Roberts 2005). The tet(w) gene encodes a protein responsible for ribosomal protection from the action of tetracycline (Chopra and Roberts 2008). ATCC 55730, the origin of the tet(W) genes, is still not clarified due to the presence of an integrase but missing mobile and transfer genes (Egervärn et al. 2009b). In addition to the tet(W) gene, the resistance gene lnu(A) for lincomycin has been detected. In 15 different phenotypic resistances, any transferability tests of the genes to other bacteria failed (Kastner et al. 2006). According to other investigations, *L. reuteri* strain ATCC 55730 does not possess any transmissible vancomycin genes, although the strain shows phenotypic resistances to vancomycins, gentamicin, ciprofloxacin, trimetophrim, ß-Lactams and others (Kastner et al. 2000; Klein et al. 2000). Table 7 summarizes the determined resistances, either plasmid-located or phenotypical.

Identification and Curing of the Plasmids

Rosander et al. (2008) aimed to remove the two resistance genes, tet(W) and lnu(A), leading to a daughter strain without the respective plasmids. The identification of the location of resistance genes was accomplished due to a draft genome sequence and subsequent BLAST search using known resistances genes (Båth et al. 2006; Rosander et al. 2008). No ß-lactam resistance genes were found in the ampicillin resistant ATCC 55730 strain, although point mutations in putative *penicillin-binding-proteins* (Pbps) were detected. These Pbps are common in many bacteria and the associated genes are located on chromosomes. Therefore, the ß-lactam resistances seem not to be transferable to other species. From 544 contigs, four were found to contain plasmid-related genes (Båth et al. 2006; Rosander et al. 2008). These contigs were arranged in plasmids, sizes 8.1 (pLR580), 12.2 (pLR581), 14.2 (pLR585) and 19.1 (pLR584). The resistance gene tet(W) has been linked to pLR581 and the gene lnu(A) to pLR585. The GC content of the removed plasmids was slightly higher, ranging from 39% for pLR581, 41% for pLR585, to the average *L. reuteri* amount of 38%.

Plasmid-curing was subsequently performed via protoplast formation techniques, followed by cell wall regeneration. The removal of the two plasmids was carried out in two consecutive steps. The bacterial cells were incubated with the respective protoplast buffer and plated on MRS plates with or without tetracycline. The same procedure was performed with lincomycin. Colonies which rejected the right plasmids (pLR581 and pLR585) did not grow with the respective antibiotics. Therefore, a selection of suitable

colonies could be identified through a polymerase chain reaction (PCR). Genes from the two other plasmids were still detectable, indicating that only the tet(W) and lnu(A) containing plasmids were removed. Finally, the double "cured" strain was named *L. reuteri*, DSM 17938.

The plasmid formation method has been promoted as a natural event, due to the spontaneous rejection of plasmids during induced stress. The likelihood of spontaneous genetic modification and induced mutation is assumed negligible (J Heimbach LLC 2008; Rosander et al. 2008). This topic will be further elaborated upon in Chapter 5.3. .

Substantial Equivalence of Strain ATCC 55730 and DSM 17938

The determination of substantial equivalence is not only important for the regulation of novel food or novel microorganism but also for the safety evaluation (see Chapter 4.3).

Substantial equivalence means that the modified strain is as safe as the comparable strain, with a long history of safe use even when one strain misses two whole plasmids (Szajewska et al. 2014). Both removed plasmids harbour 13 genes, respectively, which are believed to not be linked with any probiotic properties. *Repetitive extragenic palindromic-PCR* (repPCR) showed no difference in mother and daughter strains (Rosander et al. 2008). The repPCR technique acts as a beneficial tool for identifying the genetic fingerprint of the microorganism on the subspecies or strain level due to given sensitivity and flexibility (Ishii and Sadowsky 2009). No differences regarding the fermentation pattern, as summarized in Table 6, could be established. Furthermore, the production of reuterin, mucus binding capacity, bile tolerance and cell morphology seem to be identical for both strains. Although the growth time tested in overnight cultures of both strains has been identical, the growth density of strain DSM 17938 is comparatively significant higher. Rosander et al. (2008) explained the improved growth with the two removed plasmids and the subsequent decline in replication burden of strain DSM 17938. There is evidence that the survival rate under acidic conditions of pH 2.0 seems to improve in strain DSM 17938. According to the findings of Rosander et al. (2008), growth inhibition of *S. enterica* serovar Typhimurium, *C. difficile*, *E. sakazakii* and *C. albicans* has been observed. Both strains need glycerol as substrate for reuterin production and the subsequent pathogen inhibition exposes no difference in effectiveness. In short, the two

strains show almost identical morphological and genetically characteristics, except for the two deleted plasmid (Rosander et al. 2008).

The second question of interest is if both strains are identical with respect to their beneficial probiotic properties and health effects, in addition to mucus binding capacity. Rosander et al. (2008) conducted the first clinical placebo controlled double-blind comparison study of these two strains, involving sixteen healthy participants. The dose was administered in both *L. reuteri* groups DSM 17938 and ATCC 55730 ($8x10^8$ CFU/day, respectively) in addition to a high dose group involving $6.5x10^{10}$ CFU/day DSM 17938 and the placebo group. The *L. reuteri* administration lasted a period of 28 days. Results were achieved based on faecal and blood sampling and general health examination (weight, pulse, blood pressure, body temperature). All health parameters remained unchanged and no incidence of bacteraemia occurred. Subjects given *L. reuteri* strains DSM 17938 or ATCC 55730 showed similar levels in their faeces at all baselines, comparable to a trial conducted by Egervärn et al. (2010) and Valeur et al. (2004). Higher amounts of administered DSM 17938 also resulted in higher detectable levels in faeces, a fact which has also been noted in a trial by Savino et al. (2010). After a washout period of two weeks, no *L. reuteri* strains were detected in faeces. This indicates no steady gut colonization of the administered strains, but survival throughout the gastrointestinal tract during administration (Rosander et al. 2008).

A meta-analysis comprising five studies investigated the bioequivalence of *L. reuteri* strains DSM 17938 and ATCC 55730 in treating acute gastroenteritis in children. The few suitable studies were lacking in one or more aspects. The authors of the meta-analysis agree that a functional equivalence of the *L. reuteri* strains is feasible, but only with regard to acute diarrhoea due to similarities in reduced diarrhoea duration and an increased number of cured children by day three for both strains (Szajewska et al. 2014).

Another study compared its findings in terms of regurgitation in children linked to administration of DSM 17938 with a preceding study, which tested the same parameters using ATCC 55730. Both strains show equivalent effects in enhanced stomach emptying and reduced fasting antral area. Although the authors of study in 2010 comment that strain DSM 17938 was only used in the study due to the commercial unavailability of strain 55730 (Indrio et al. 2008; Indrio et al. 2011).

Two other comparable studies investigating the activity of *L. reuteri* on breastfed colicky infants, showed similar outcomes. The randomized, controlled double-blind study, conducted by Savino et al. (2010) compared the effects of strain DSM 17938 with a placebo administration. Whereas, the comparable open trial of 2007 observed the outcomes of strain ATCC 55730 in comparison to medical treatment (simethicone). Both strains resulted in a positive impact on crying time and symptoms (Savino et al. 2010; Savino et al. 2007).

To sum up, a functional equivalence in the treatment of acute diarrhoea, regurgitation and colic in infants seems likely. Although the effects of single strains were not investigated in all clinical studies.

5.2. Safety Status of *Lactobacillus reuteri* strain DSM 17938

It should be stressed that the FDA did not question two proposals for *L. reuteri* strain DSM 17938, which is added to food as well as to infant formula. The modified strain has gained GRAS status in the USA (J Heimbach LLC 2008; 2011). In Europe, EFSA has not questioned the QPS safety status of *L. reuteri* in its updates, and has not considered a potential safety gap for strain DSM 17938 (EFSA 2007; 2013). It is commonly believed that *L. reuteri* ATCC 55730 does not harm human health, even in immunocomprised individuals (Casas and Dobrogosz 2000; Connolly 2004; Indrio et al. 2008; Savino et. al 2007; Wolf et al. 1995; 1997). Numerous studies conducted worldwide, which have administered strains DSM 17938 to infants or adults, have not noted obvious adverse health effects in healthy individuals, or in those suffering from ongoing infection (Coccorulla et al. 2010; Dinleyici and Vandenplas 2014; Francavilla et al. 2012; Mangalat et al. 2012; Rosander et al. 2008; Savino et al. 2010; Valeur et al. 2004). Nevertheless, the following section will deal with available literature regarding this subject and question the potential pathogenicity of strain DSM 17938 particularly according to the European QPS system.

Body of Knowledge

The body of knowledge comprises history of use, level of exposure, relevant scientific literature, clinical trials and industrial application (see also Chapter 4.2.).

- History of use, exposure and industrial application

The species *L. reuteri* was first described in the 1980 by Kandler et al., but had been known long before as *L. fermentum* IIb *(*Klein et el. 1998). It is assumed that *L. reuteri* is the dominant autochthonous species in the human gastrointestinal tract (Reuter 2001). *L. reuteri* strain ATCC 55730 has been sold since 1991 as a functional ingredient in food. Dietary supplements containing ATCC 55730 have been on the market since 2000, mainly distributed by BioGaia® and partners (Biogaia 2014). In 2004, more than 200 million doses of 10^6 cfu were sold worldwide without reported health effects (Connolly 2004). In 2007 strain ATCC 55730 was replaced by the modified strain DSM 17938, which is now commercially available in up to 85 countries, with Europe as its' greatest market (Biogaia 2014). Originally, BioGaia wanted to add strain DSM 17938 to processed cheese, yogurt, ice cream, fruit juice/drinks, vegetables, beverage bases, energy bars and chewing gum (J Heimbach LLC 2008). Currently, strain DSM 17938 is available in supplemented dairy products such as milk and yoghurts for infants, infant formula, drops, straws, powder, chewable tablets and capsules (see Table 8) (BioGaia 2014). Each product contains an average amount of 10^9 cfu/serving to guarantee a minimal level of 10^8 cfu per ingested serving of viable bacteria. Therefore, the maximum "estimated consumer exposure from these intended uses is less than 10^9 to 10^{10} cfu/day" (J Heimbach LLC 2008, pp. 1).

Table 8: BioGaia products available or containing BioGaia Lactobacillus reuteri strains

Product	**Country**	**Dose (cfu/d)**	***L. reuteri* strains**
BioGaia ProDentis lozenges and co-branding	*Belgium, Bulgaria, Canada, Czech Republic, Denmark, Finland, Indonesia, Luxembourg, Poland, Singapore, Slovakia, USA*	2-4x10^8	DSM 17938 and ATCC PTA 5289
BioGaia ProTectis chewable tablets and co-branding	*Belgium, Bolivia, Botswana, Brazil, Bulgaria, Canada, Chile, Croatia, Denmark, France, Germany, Greece, Hong Kong, Hungary, Indonesia, Ireland, Israel, Italy, Japan, Latvia, Lithuania, Luxembourg, Pakistan, Peru, Poland, Portugal, Rumania, Slovakia, Slovenia, Spain, Taiwan, Turkey, UK, Ukraine, USA*	1-2x10^8	DSM 17938
BioGaia ProDentis drops	*Singapore*	2x10^8	DSM 17938 and ATCC PTA 5289
BioGaia Protectis drops and co-branding	*Armenia, Austria, Azerbaijan, Belarus, Belgium, Bolivia, Bulgaria, Canada, Chile, Colombia, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Georgia, Germany, Greece, Hong Kong, Hungary, Indonesia, Ireland, Israel, Italy, Japan, Kazakhstan, Latvia, Lesotho, Lithuania, Luxembourg, Malaysia, Mexico, Namibia, Pakistan, Peru, Poland, Portugal, Puerto Rico, Romania, Russia, Serbia, Singapore, Slovakia, Slovenia, South Africa, South Korea, Spain, Swaziland, Switzerland, Taiwan, Turkey, UK, Ukraine, USA*	1x10^8	DSM 17938
BioGaia probiotic straw and co-branding	*Italy, Japan, USA*	1x10^8	DSM 17938
BioGaia Oral Rehydration Solution and co-branding	*Spain, Portugal, Finland, Greece, Italy, Poland, Portugal, Slovakia, Spain, Ukraine*	>10^9	DSM 17938
Infant Formula	*Australia, Bangladesh, Belgium, Burma, Cambodia, Chile, Colombia, France, Germany, Greece, Guatemala, Indonesia, Iran, Israel, Italy, Jordan, Laos, Lebanon, Malaysia, Mexico, Pakistan, Peru, Philippines , Poland, Portugal, Romania , Saudi Arabia, Serbia, Singapore, South Africa, Spain, Sri Lanka, Thailand, USA*	10^8 *	
CasenBiotic Reuteri sachet	*Portugal, Spain*	1x10^8	DSM 17938
BioGaia Tablets and co-branding	*Australia, Botswana, Estonia, Finland, Lesotho, Malaysia, Namibia, New Zealand, Russia, South Africa, South Korea, Swaziland*	1-2x10^8	DSM 17938
BioGaia Gastrus	*Future Launch*		DSM 17938 and ATCC PTA 6475.
BioGaia ProDentis chewing gum	*Future Launch*		DSM 17938 and ATCC PTA 5289

(BioGaia 2014)

* $1.35x10^7$ cfu/ml = 800ml target ingestion = 10^8 cfu/day exposure information from J Heimbach LLC(2011)

Having the largest market for probiotics worldwide, Europe is most exposed to *L. reuteri* strain DSM 17938, followed by Asia (see Figure 6). Besides the daily consumption of functional food and supplemented products enriched with *L. reuteri* strain DSM 17938, natural exposure to the mother strain ATTCC 55730 (coming from the gut, breast milk and natural surroundings) should be not underestimated (Casas and Dobrogosz 2000; Sinkiewicz and Ljunggren 2008; Rosander et al. 2008; Singh et al. 2012).

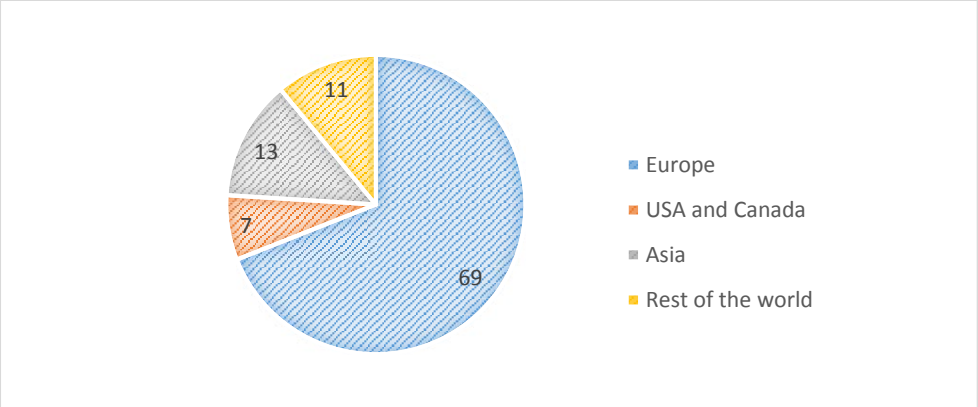

Figure 6: Sales by geographical market of BioGaia products (BioGaia 2013)

According to the BioGaia GRAS proposal, the production of strain DSM 17938 is carried out by different suppliers and can vary according to the methods used (J Heimbach LLC 2008). Even minor deviations in production procedures (incubation temperature, pre-treatment methods, growth media) and the source of the probiotic itself could influence the outcomes of conducted clinical studies and bacterial properties (Grześkowiak et al. 2011; 2014). If it also influences the in vivo properties of *L. reuteri* strains ATCC 55730 and DSM 17938 is still unclear (Urbanska and Szajewska 2014). In the case of *L. reuteri* DSM 17938, the source of the strain seems to always be the same since the frozen cultures are deposited in working cell banks. It is noted that all suppliers comply with good laboratory practices, but differ in cyroprotective coating details when writing proposals. The subsequent harvesting of cells is carried out in their stationary phase, which has been shown to have a positive impact on bacterial adhesion activity in Caco-2 cells (Deepika et al. 2009; J Heimbach LLC 2008). Specification parameters are defined for all suppliers identically, including microbiological purity, content of heavy metals, *L. reuteri* activity, strain identity (determined by 16sRNA gene analysis) and

product specification. Pure bacteria are then controlled, stored and distributed at a temperature no higher than -18°C and with a shelf life of 24 months (J Heimbach LLC 2008). Besides factors such as growth media, the final food matrix can also influence the probiotic properties of *Lactobacillus* strains (Dommels et al. 2012; Hüfner et al. 2008; Kankaanpää et al. 2011).

- Scientific literature

To assess the body of knowledge, literature research was conducted mainly using two databases, Scopus and PubMed. Both searches resulted in similar outcomes at the date of the search (06 May, 2014). The results of the process are displayed in Table 9 below. Google Scholar, as an unofficial scientific database, served as a source of comparison. Only articles and works obtained by Scopus, PubMed and related databases were evaluated and reported in this work. As summarized in Table 9, the search for both *L. reuteri* strains resulted in a similar number of hits. Strain ATCC 55730 yielded significantly more results than DSM 17938. Species *L. reuteri* is widely mentioned in scientific literature and enjoys common knowledge status, whereas the relative new strain DSM 17938 has been mentioned frequently in scientific papers since 2012. Clinical studies are available for both strains and will be discussed with respect to pathogenicity in the next chapter.

Table 9: Results of all searches regarding Lactobacillus reuteri in different databases (6. Mai 2014).

Database	**Search term**	**Hits (in article, title, abstract, keywords)**	**Hits (in title)**
Scopus	*Lactobacillus reuteri* DSM 17938	48	18
	L. reuteri (DSM) 17938	3 (37)	1(1)
	Lactobacillus reuteri ATCC 55730	54	18
	L. reuteri (ATCC) 55730	44 (48)	1
	Lactobacillus reuteri	1478	475
	Lactobacillus reuteri SD2112	13	0
PubMed	*Lactobacillus reuteri* DSM 17938	45	
	L. reuteri (DSM) 17938	39	
	Lactobacillus reuteri ATCC 55730	46	
	L. reuteri (ATCC) 55730	41	
	Lactobacillus reuteri SD2112	9	
Google Scholar	*Lactobacillus reuteri* DSM 17938	531	
	Lactobacillus reuteri ATCC 55730	1.610	
	Lactobacillus reuteri SD2112	366	

- Pathogenicity and Clinical Studies

This work will not question the safety of *L. reuteri* in general, since EFSA included the strain in all of its QPS updates and has not questioned its safety status (EFSA 2007; 2008a; 2009b; 2010; 2011b-2014).

L. reuteri strain ATCC 55730 is a well-studied probiotic with promising health effects (Casas and Dobrogosz 2000; Connolly 2008; Indrio et al. 2012; Di Nardo et al. 2014; Savino et al. 2007; Valeur et al. 2004; Wolf et al. 1995; 1998). The first clinical study investigating possible beneficial or adverse effects of strain ATCC 55730 in healthy male subjects concluded a good tolerance of doses up to 10^{11} cfu/day administered for 21 days. Colonization of the human gut was maintained for at least seven days after termination (Wolf et al. 1995). A similar study summarized no adverse safety events and a lack of tolerance problems in patients infected with HIV (Wolf et al. 1998).

The meta-analysis conducted by Connolly (2004) reviewed and evaluated eleven clinical trials carried out with strain ATCC 55730. These trials were conducted on new-borns, infants, children, and susceptible and healthy adults, receiving different doses of 10^5 up to 10^{11} cfu/days. Clinical safety and favourable tolerance were detected in all studies.

As part of a comprehensive study, pregnant woman with a history of allergies received strain ATCC 55730 until the date of delivery. Their babies continued ingesting the strain until 12 months. No subjects showed health or developmental abnormalities (Abrahamsson et al. 2007).

Based on several clinical studies on healthy and susceptible subjects, strain ATCC 55730 can be categorized as likely safe for human consumption. The safety of strain ATCC 55730 established the basis for the daughter strain, DSM 17938, but with limitations. It is important to assess if the removal of two plasmids negatively affects the safety of the daughter strain. Table 10 summarizes a great variety of recently conducted studies, investigating the administration of L.reuteri DSM 17938.

A small meta-analysis of randomized controlled trials in 2014 concluded that the strain DSM 17938 might be effective in reducing or preventing gastrointestinal disorders in children as regurgitation, colic and diarrhoea. No adverse health effects were reported

among non-immunocomprised children in the included studies (Urbanska and Szajewska 2014).

One study addressed the safety and tolerance of strain DSM 17938 in adults. Surprisingly, the number of mild vomiting episodes and nausea were higher in the probiotic group than in the placebo group. The authors concluded that the reported effects were not directly linked to probiotic consumption (Mangalat et al. 2012).

The most recent study in 167 infants, reported a higher fussing time in the probiotic group when compared to the placebo group (Sung et al. 2014). No other clinical study reported adverse effects, even when carried out on highly susceptible population groups, such as pre-term neonates and ill infants (Dinleyici and Vandenplas 2014; Hunter et al. 2012; Rojas et al. 2012). Further studies of administered *L. reuteri* strain DSM 17938 reported no impact on the physical development of infants, including body weight, length, and head circumference (Indrio et al. 2011; Savino et al. 2010).

A community-based trial gives some evidence that strain DSM 17938 is also well tolerated in remote areas, where the population has not been exposed daily to probiotic products and enriched functional food (Oberhelman et al. 2014). Drawing on the current state of knowledge, it is likely that *L. reuteri* strain DSM 17938 possesses the same safety status as its mother strain with respect to clinically adverse events.

Table 10: Summary of trails, investigating the effects of administered Lactobacillus reuteri strain DSM 17938

Clinical trials	Aim	Study Design*	Subjects	Dose (cfu/d)	Safety-related results
Dinleyici and Vandenplas (2014)	Effects on diarrhoea/acute gastroenteritis	RD, SB, Multicentre	64 hospitalised children	10^8 for 5 days	Only favourable outcomes, no adverse health effects
Francavilla et al. (2014)	Effects of *L. reuteri* DSM 17938 together with L. reuteri ATCC PTA 6475 in Helicobacter pylori infection	RD, DB, PC	100 *H. pylori* positive naive patients	2×10	Only favourable outcomes, no adverse health effects
Gutierrez-Castrellon et al. (2014)	Effects on frequency and duration of diarrheal episodes and other health outcomes	RD, DB, PC	168 day school healthy children	10^8 for 3 months	Only favourable outcomes, no adverse health effects
Oberhelman et al. (2014)	FDA Phase I trial in in the Peruvian Amazon	Community based trial	45 healthy adults in Amazonas area	10^8 for 5 days	No significant outcomes , no evidence of invasive

					infection resulting from the probiotic
Sung et al. (2014)	Effects on crying and fussing time	RD, DB, PC	167 ill breastfed infants or formula fed infants	10^8 for 1 month	No significant outcomes, no adverse health effects reported by parents, longer fussing time in probiotic group than in placebo group
Dore et al. (2013)	Effects on *H. pylori* eradication therapy	Open Label, single centre	22 *H. pylori* positive patients	10^8 plus pantoprazole (40 a day for 8 days)	Favourable effects are likely, no adverse health effects
Szajewska et al. (2013)	Effects on infantile colic	RD, DB, PC	80 infants with colic	10^8 for 21 days	Only favourable outcomes, no adverse health effects
Hunter et al. (2012)	Effects on necrotizing enterocolitis rate in neonates (birth weight ≤ 1000g) after receiving *L. reuteri* prophylaxis	retrospective cohort study	311 neonates (from these 79 received *L. reuteri*)	~0.5-1 x 10^8 for up to 2 years	Favourable effects are likely, no adverse health effects
Mangalat et al. (2012)	Effects on safety, tolerance and immune modulation	Prospective RD, DB, PC	40 healthy adults	5×10^8 For 2 months	Favourable effects are likely, safe and well tolerated in adults (adverse health effects are not related to *L. reuteri*)
Rojas et al. (2012)	Effects on infant mortality and nosocomial infections	RD, DB, PC	750 preterm infants	10^8 until death or discharge	No significant outcomes , trends suggest a protective role for mortality, no adverse health effects
Wanke and Szajewska (2012)	Effects on the prevention of nosocomial diarrhoea	RD, DB, PC	106 hospitalised children	10^8 for the duration of hospital stay	No significant outcomes, no adverse health effects
Indrio et al. (2011)	Effects on regurgitation frequency and gastric emptying time	RD, DB, PC	42 healthy infants	10^8 for at least 30 days	Only favourable outcomes, no adverse health effects
Coccorullo et al. (2010)	Effects on functional chronic constipation	RD, DB, PC	44 infants with chronic constipation	10^8 for 8 weeks	Only favourable outcomes, no adverse health effects
Savino et al. (2010)	Effects on infantile colic and on gut microbiota diversity	RD, DB, PC	50 exclusively breastfed colicky infants	10^8 for 21 days	Only favourable outcomes, well tolerated, no adverse health effects
Vivekana et al. (2010)	In vivo antimicrobial effects of *L. reuteri* Prodentis (DSM 17938 & ATCC PTA 5289) on chronic periodontitis	RD, DB, PC	30 adults	10^8 for for 42 days	Favourable effects are likely, no adverse health effects

**RD: randomised; DB double blind; SB: single blind; PC: placebo controlled*

A comprehensive report evaluated the safety of probiotics by reviewing 622 studies. Adverse events, such as bacteraemia, were rarely reported. The included studies, which tested *L. reuteri* strain DSM 17938 and ATCC 55730, documented no adverse and unexpected side effects (Hempel et al. 2011).

To evaluate the impact of strain DSM 17938 on the microbiota of colicky infants, faecal samples were analysed by DNA sequencing of 16 S rRNA genes, and PCR amplified. Twenty-nine infants received 10^8 cfu daily during 21 days. With the help of 454 pryosquencing, the main phyla in infants' intestine could be assessed as following: *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes*. However, administered doses of *L. reuteri* DSM 17938 did not affect the microbiota in the study by Roos et al. 2013. Infants who were characterised as responders (<50% reduced crying time) had increased *Bactericides* levels at the end of the study in comparison to non-responders. Based on these outcomes, it can be assumed that strain DSM 17938 does not lead to an unfavourable imbalance of gut microbiota in infants. On the contrary, this outcome indicates a shift of possible preceding microbiota imbalance towards a more favourable diversity (Roos et al. 2013).

To support this theory, an in vivo study in CD1 neonatal mice gave evidence that the *L. reuteri* strain obviously enhances growth, crypt height and migration of enterocytes. The study also showed that strain DSM 17938 is able to support the phylogenetic diversity of gastrointestinal microbiota. Any changes and remodelling occurred after 1 day but only lasted for the length of administration, which has also been supported by several other human studies (Egervärn et al. 2010; Preidis et al. 2012; Rosander et al. 2008).

The lack of ascertained permanent gut colonization might reduce the likelihood of potentially adverse effects by bacterial overgrowth (Connolly et al. 2005). Nevertheless, there are still unanswered questions concerning *L. reuteri* strain DSM 17938, such as the mechanism of effect or long-term consequences of use (Chumpitazi and Shulman 2014). Concerning the latter, encouraging results are given by Hunter et al. (2012). In this study the length of administration of strain DSM 17938 was longer than in most other study approaches, with no reported study-related adverse events.

As mentioned in Chapter 5.1, O'Sullivan carried out a draft genome sequence of strain DSM 17938. The results emphasize that no required virulence genes or clusters are part of the genome, except one gene that could encode haemolysin.

Haemolysin is a virulence factor of several pathogens, such as *of Staphylococcus aureas* and pathogenic *E. coli* strains (Karginov et al. 2007; Segar et al. 2014). These genes have also been found in other sequenced Lactobacillus strains regarded as safe, such as *L. reuteri* strain ATCC 55730 (NCBI n.d.b). Thus, it is likely that the suspicious gene of strain DSM 17938 does not contribute to pathogenic behaviour (O'Sullivan 2008, p.109-118). It is important to know if the removed plasmids host any genes which would negatively influence safety in their absence. Therefore, Rosander et al. (2008) summarized the genes of the removed plasmids linked to their functions. Besides the tetracycline resistance protein W13, plasmid pLR581 contains genes mainly for replication, transcription regulation, DNA integrase/recombinase, arsenate and arsenite efflux pumps, and phage-related proteins. The plasmid pLR585 hosts the lincomycin resistance gene in addition to 13 genes, such as for polyketide antibiotic exporter. None of these genes seem to be relevant to the general safety status of the strain (Rosander et al. 2008).

In terms of safety, other parameters such as adhesion to intestinal cells, mucin degradation, lactic acid production and antibiotic resistances should be considered. Most points have been elaborated upon in Chapter 5.1. Excessive mucin degradation which can weaken intestinal barrier function is not reported in the case of *L. reuteri* strain DSM 17938 or the mother strain ATCC 55730 (Ouwehand et al. 2002, Rosander et al. 2008).

Adhesion capability is necessary for probiotics to trigger beneficial effects in the human body, also contributing to infectivity (Kirjavainen et al. 1999). As mentioned in Chapter 5.1, the adhesion properties of strain DSM 17938 are weaker than in related strains (Jensen et al. 2012). To support these findings, Rosander et al. (2008) compared adhesion properties of the 'safe' strain ATCC 55730 with DSM 17938, with no differences detected.

A minor possibility exists that enhanced D-lactate production could lead to lactic acidosis, especially in infants. During a long-term study, infants received *L. reuteri* strain ATCC 55730 for 12 months, and no increased D-lactate levels were measured (Connolly et al., 2005). In the case of the two *L. reuteri* strains DSM 17938 and ATCC 55730, the main product is L-lactic acid and not the stereoisomer D-lactic acid. The latter is produced in patients suffering from small short bowel where *Lactobacillus* ssp. produce D-lactic acid from easily fermentable carbohydrates, followed by shifts of bicarbonate, base

excess, anion gap and hyperventilation (Bongaerts et al. 2000). In healthy subjects, no direct health problems linked to D-lactic acid producing *L. reuteri* strains have been reported. Strain ATCC 55730 is one of the real indigenous bacterium, therefore a certain amount of DL-lactic acid production in the human gut is from birth on a natural event. A preceding bacterial overgrowth is necessary to produce D-lactic acid in harmful amounts, but to date no documented evidence for *L. reuteri* strain DSM 17938 overgrowth has been reported in literature (Connolly et al. 2005). Inclusive clinical studies and their outcomes tend to assume overall safety of L. reuteri strain DSM 17938

- Antibiotic resistance and gene transfer

As part of Chapter 5.1 the antibiotic resistance patterns of *L. reuteri* strain ATCC 55730 have been already discussed and described. Anyway, it is important to know if strain DSM 17938 carries any antibiotic resistances beside the two cured plasmids.

As part of the same work, Rosander et al. (2008) removed the two antibiotic resistance carrying plasmids of the mother strain ATCC 55730 and subsequently tested the resulting strain DSM 17938 for any remaining resistances. Not surprisingly, the MIC levels for tetracycline and linyomcin were in a normal range as displayed in Table 11.

. This indicates the direct link of the two plasmids to the antibiotic resistances (Egervärn et al. 2009; Rosander et al. 2008). In the study by Egervärn et al. (2010) the transferability of tetracycline resistance gene W to other *Lactobacillus* strains, as well *Bifidobacterium* and *Enterococcus* strains was tested. The study participants received either the strain ATCC 55730 or DSM 17938 (control group) in equal amounts for 14 days ($5x10^8$ cfu/day). At the end of the study, DNA was extracted from all faecal samples and analysed for the reuteri-tet(w) gene by PCR method. The samples of the subjects receiving the strain ATCC 55730 produced positive signals for the tet(w) gene, whereas no signal could be detected in the control group. These results support an efficient curing of tet(w) gene carrying plasmid. No events of tet(W) transfer from strain ATCC 55730 and DSM 17938 to other gut bacteria could be detected (Egervärn et al. 2010). Whereas all other intrinsic and phenotypic resistances described are still maintained in strain DSM 17938 (Egervärn et al. 2009; Kastner et al. 2006¸ Rosander et al. 2008).

Table 11: MIC levels of the Lactobacillus. reuteri strains ATCC 55730 and DSM 17938 (Rosander et al. 2008)

Strain	*MIC (µg/ml)*		
	Tetracycline	Lincomycin	Clindamycin
ATCC 55730	>256	>16	<0.125
DSM 17938	12-16	0.25	<0.125

5.3. Plasmid-Curing as Genetic Modification – Opinions of Experts

This chapter deals with the question of whether or not the applied plasmid-curing at *L. reuteri* strain ATCC 55730 results in a genetically modified daughter strain. For this, a number of experts in lactic acid bacteria research were consulted by asking them three simplified questions. The given answers from six experts from different countries are summarized and discussed for any commonalities or significant differences in the following subchapters. In order to keep their anonymity, the names were arbitrarily designated as experts 1 to 6.

Question 1

Is the deliberate removal of antibiotic resistance carrying plasmids considered as genetic modification in bacteria used in a food product?

The majority disagrees with the statement that the applied plasmid-curing technique falls under genetic modification, arguing that something similar could also occur naturally as an evolutionary event (Expert 1;2;5;6 2014). The genetic modification defined techniques by the Directive 2009/41/EU Annex I, Part A are important to consider:

*1. "*Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation*".*

*2. "*Techniques involving the direct introduction into a micro-organism of heritable material prepared outside the microorganism, including micro-injection, macro-injection and micro-encapsulation*".*

*3. "*Cell fusion or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally*".*

The Directive excludes techniques where no foreign (heterologous or recombinant) DNA is introduced to the cell during the process and those, which can also occur naturally, such as conjugation, transduction or transformation (see Annex I and II) (European Commission 2009a). However, the view can be different while taking into consideration other national laws (Expert 2; 4 2014). For instance in Canada, any genetic change deliberately induced, is defined as a genetic modification, and falls under the regulations of a novel food or ingredient that must go through the following evaluation process:

"An organism, such as a plant, animal or bacterium, is considered genetically modified if its genetic material has been altered through any method, including conventional breeding*"* (Health Canada 2012).

Therefore, a global clear clarification of this issue is hard to provide, as different national laws give different definitions of genetic modification. Under European law, the removal of plasmids by protoplast formation (Directive 2009/41/EC Annex I and Annex II) is not defined as genetic modification, as no new genetic material is introduced into the strain ATCC 55730. Moreover, the loss of the plasmid could also occur under natural conditions (Expert 4 2014; Stanisich 1988). Therefore, the resulting daughter strain DSM 17938 is not considered as a GMM according to the European regulatory approach (European Commission 2009a). However, the possibility remains to categorize plasmid-curing as genetic modification, but as a "natural" acceptable form (Expert 3 2014). Questions 2 will discuss the topic further.

Question 2

Do you think that the methodology/technique facilitating the removal is crucial for being able to answer the above mentioned question?

Almost all experts emphasize that it might be important to know the applied methodology and techniques except under Canadian law (see Question 1) (Expert 1;2;3;4;5;6 2014).

A process which simulates a possible natural event in an ecological niche representing the natural habitat of strain ATCC 55730 is considered not a genetic modification of bacteria under European law (Expert 5 2014). Plasmidless segregants can also occur naturally because of defects in replication mechanisms (Stanisich 1988, pp.11-48). Conditions in food such as, as temperature and the passage through the gastrointestinal tract can initiate stress, leading to the "natural" loss of plasmids (Expert 4 2014).

However, Directive 2009/41/EC distinguishes between those methods which involve the use of recombinant-nucleic acid molecules and those which do not. Normally, plasmid-curing techniques (see Question 3) do not introduce foreign DNA into the bacterial cell and subsequently they are not literally a genetic modification method according Directive 2009/41/EC (European Commission 2009a). The situation is more complicated when foreign genetic material is introduced into the host cell. For instance, an engineered plasmid used for facilitating the removal of the host plasmid (Plasmid incompatibility method) (Expert 2;6 2014; Liu et al. 2012; Ni et al. 2008). Some plasmid-curing methods involve DNA intercalating compounds which are known to induce spontaneous and unpredictable mutations, as e.g. ethidium bromide (see Question 3) (McCann et al. 1975; Stanisich 1988, pp.11-48).

Similar to Question 1, an answer depends on the stringency of national law and the subjective attitudes towards this issue.

In Canada any alteration of the genetic material through any method is considered a genetic modification, therefore the answer might be under Canadian law: "The technique facilitating the plasmid-curing is not crucial, as all kinds of techniques and methods are considered genetic modification" (Expert 4 2014; Health Canada 2012).

In other countries, particular attention must be paid to the fact that if applied plasmid-curing technique influences the DNA in a way that is defined as genetic modification under the applicable national or European law.

Question 3

Among the techniques used for the above mentioned purpose, which one would you consider as the most suitable one in order to secure the safety and the usability of the strain in food? Common plasmid-curing techniques, used in protocols, are listed below:

a. *Chemical agents such as acridine orange, ethidium bromide¸ acriflavine, and sodium dodecyl sulfate*
b. *Protoplast formation*
c. *Physical curing treatments :*
    i. *High-temperature curing*
    ii. *Low-temperature curing*
    iii. *Treatment with UV-light*
d. *Methods based on plasmid incompatibility*
e. *Electroporation?*

Answers to this question depend on the expert's focus. One would consider more the regulatory background of the country and not the scientist's viewpoint and experience. However, to give a scientifically correct answer, *"suitability*" should be defined as follows (Expert 4 2014):

Suitability, in terms of this question, is a process that leads to a minimum impact on the genome of the strain and subsequently does not lead to a product that has:

- *adverse effects on human health, animal health or the environment*
- *and differs from the food which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for the consumer*

The safety of the strain is not necessarily correlated with the safety of the product. Nevertheless, according the law, the safety of the strain is the basis for using it in food products (according Article 4(1) of Regulation (EC) No 1829/2003) (European Commission 2003b).

Theoretically, all treatments are considered suitable for accomplishing the intended aim, although some initiate more likely mutations and cell damage than others do (Expert 6 2014).

For instance, plasmid-curing protocols using chemical agents, such as acridine orange, ethidium bromide and acriflavine or UV-treatments, are able to induce secondary mutations in the host chromosome and effect other strain characteristics than the plasmid elimination alone can achieve (Expert 1;2;3;4;6 2014; Liu et al. 2012). SDS, being non mutagenic, is suitable for *Lactobacillus* ssp. as well, but is not recommended by most addressing this issue (CIR Publication 1983; Expert 1 2014; Lavanya et al. 2011).

Techniques such as electroporation or plasmid incompatibility might be within a grey area between conventional techniques and genetic modification, depending on if genetically modified plasmids or markers are used and introduced (see Question 2) (Expert 1 2014; Liu et al. 2012; Ni et al. 2008). However, in Canada, methods such as incompatibility and electroporation are not accepted techniques for food purposes at all (Expert 4 2014; Health Canada 2012).

To sum up, the majority recommends those techniques showing a lower risk of introducing mutations, protoplast formation and low temperature curing (Expert 1;2;3;4;6;5 2014). Table 12 summaries plasmid-curing techniques valued according priority of acceptance and probability. The effect of every single technique also depends on the strain properties and robustness and is therefore not easy to predict. A resequencing step of the derivative for selecting the one that shows the best predicted performance is therefore recommended (Expert 6 2014). Overall, regardless of which technique or method has been selected, the functionality and the characteristics of the strain must remain unchanged in the plasmid-cured strain (Expert 2 2014).

Table 12: Plasmid-curing techniques valued according priority of acceptance and probability of working with respect to food derived by the experts' opinions (Expert 6 2014)

	Priority of Acceptance	**Probability of working**
Chemical agents	Questionable	Good
Protoplast formation	High	Good
High temperature curing	High*	Good
Low temperature curing	High	Lower
UV light treatment	Medium	High
Methods based on plasmid incompatibility**	Depending	
Electroporation**	Depending	

* Heat can induce mutations (Expert 4 2014; Liu et al. 2012)

**The use of incompatible plasmids requires an electroporation step (Expert 6 2014)

Conclusions of Chapter 5

Under European law, plasmid-curing techniques are not considered genetic modifications, although some techniques are within a grey zone (chemical agents, UV light, plasmid incompatibility…). It is important to emphasize that each country or community has its own regulations and each scientist its own approach to address this issue. Physical treatments, as well protoplast formation, are highly recommended for the plasmid-curing purpose, as they do not introduce secondary mutations. In general, all experts took the same view in order to categorize plasmid-curing as non-genetic modification, or at least as a "natural" acceptable one. Only the consulted expert from Canada emphasized that plasmid-curing utilized by any technique and method is addressed as genetic modification under Canadian law.

6. The Regulatory Grey-Zone of *Lactobacillus reuteri* Strain 17938

6.1. Regulation in Case of a Genetically Modified Microorganism under EU Law

This chapter deals with the hypothesis that *L. reuteri* DSM 17938 is a genetically modified strain under the definition laid down in Directive 2009/41 Article 2(b) (see Chapter 4.4) (European Commission 2009a). The issue whether the plasmid-curing technique leads to an unnatural change of genetic material has been discussed in the previous Chapter 5.3.

For GMM and conventional microorganisms, the same safety status applies under Regulation (EC) 178/2002: "The product must be safe for human consumption, for animals and the environment" (European Commission 2002d). Products containing or consisting of viable GMMs fall under the Regulation (EC) No 1829/2003 and/or Directive 2001/18/EC, as explained in Chapter 4.4. (European Commission 2001a; 2003b). The Directive specifically covers the environmental safety assessment (European Commission 2001a). Whereas, in case of *L. reuteri* DSM 17938 the "one door-one key" principle under Regulation (EC) No 1829/2003 might apply. This Regulation embraces the environmental safety assessment as well as the assessment of the food product itself (European Commission 2003b).

If a company attempts to commercialise the strain DSM 17938 as a GMM, a comprehensive application must be submitted to the EFSA. EFSA evaluates all the available data and prepares a scientific opinion for the European Commission. The Commission decides with the help of the member states if *L. reuteri* DSM 17938 authorisation is suitable (European Commission 2001a; 2003b). A guidance document (GMM guidance) which aims to assist the preparation of the required safety application according Regulation (EC) No 1829/2003 (EFSA 2011a) is available. According to the internationally accepted comparative approach, strain DSM 17939 as a genetic modified variant, shall be compared with the conventional counterpart on a case-by-case basis. The assessment must address all emerging direct, indirect and long-term effects relative to the counterpart (Codex Alimentarius 2003, EFSA 2011a).

Firstly, the well known, intensively studied and regarded as safe, mother strain ATCC 55730 of DSM 17938 has gained QPS status. Secondly, a product containing viable cells of the *L. reuteri* strain DSM 17938, e.g. a yoghurt supplemented with this strain, would fall under Category 4 of the GMM guidance:

"Products consisting of or containing GMMs capable of multiplication or of transferring genes" (EFSA 2011a, pp.7). This category requires the most complex assessment, since all deviations with respect to the counterpart must be documented. However, due to the fact that the counterpart gained QPS status, the information about the safety status can be less extensive than is usually warranted (Aguilera 2013; EFSA 2011a). In addition, a product containing viable modified *L. reuteri* DSM 17938 cells must clearly indicate the genetic modification on its label according to the Regulation (EC) VO 1830/2003 (European Commission 2003c). Table 14 summarizes all the information and data that would be theoretically required by the GMM guideline and their status of fulfilment. All information that is still outstanding is marked as "not available" or "partially available" since it has not been covered by this work or scientific literature.

L. reuteri strain DSM 17938, characterized as a GMM, would be the first approved genetically modified bacterium in food products in the EU (Aguilera 2013; European Commission n.d.a).

6.2. Regulation in Case of a Conventional Microorganism under European Law

In comparison to the "genetically modified theory", the definition of *L. reuteri* strain DSM 17938 as a probiotic is well established and accepted. However, the regulatory approach in terms of a conventional microorganism is as complex as a GMM, since no specific European legislation for probiotics as food supplements exists. In general, probiotics profit from a well-established history of safe use, since they do not require a pre-market safety assessment. However, probiotics have to comply with a number of European laws (Herody et al. 2010).

Due to the fact that the species *L. reuteri* has had QPS status since 2007, the strains DSM 17938 and ATCC 55730 automatically seem to be safe as well. The Scientific Committee has not questioned this so far. Nevertheless, the QPS status has no legal implication (EFSA 2007; 2013).

Besides the QPS approach, the NFR approach also applies. The strain DSM 17938 was not placed on the market before 1997, but its addition to food products intended for human consumption is legal with respect to the proven substantial equivalence and the QPS status. The substantial equivalence to the mother strain ATCC 55730 is assumable, therefore a reduced application dossier would be sufficient (see Chapter 5.1) (European Commission 1997b).

The "novel food application" has been partially fulfilled by this work, with the QPS system as a useful tool for preparing the dossier in terms of safety (Herody et al. 2010).

Health claims under the Regulation (EC) No 1924/2006 play an important role by promoting strain DSM 17938 as a probiotic (European Parliament 2006). However, no health claims including *Lactobacillus* ssp. have been authorised in the EU so far, except as "live yoghurt cultures" as explained in claim type Art.13(1) (European Commission 2014). Therefore, it is not very likely that a claim specifically for *L. reuteri* strain DSM 17938 will be authorised in the near future.

Additionally, probiotics added to organic food are not considered relevant due to their negligibly low occurrence (Herody et al. 2010).

The labelling of the product including probiotic cells of strain DSM 17938 must comply with the general labelling Directive (EC) No 2000/13, meaning that the probiotic must be indicated with its specific name in the list of ingredients (European Commission 2000a; Herody et al. 2010). In case of authorised health claims, additional labelling provisions under Regulation (EC) No 1924/2006 apply (European Parliament 2006). Table 13 illustrates and summarizes the most important European legislations which must be considered in terms of *L. reuteri* DSM 17938 as a GMM or a conventional probiotic.

Table 13: European regulatory approach for genetically modified and conventional variant of Lactobacillus reuteri DSM 17938 (see Chapter 4 for details)

Genetically Modified	**Condition of use**	**GMM Guideline**	**Regulation (EC) No**	**Directive**
DSM 17938	(EC) No 178/2002 Product contains viable cells (probiotic cultures)	Category 4	1829/2003 1830/2003 641/2004 65/2004 1946/2003*	2001/18/EC 2009/41/EC*
Conventional		**QPS**	**Regulation**	**Directive**
DSM 17938		QPS status	258/97 1924/2006 767/2009	2000/13

*If applicable

Table 14: Requirements according to the GMM guideline in terms of Lactobacillus reuteri DSM 17938, which have been already fulfilled (EFSA 2011a)

Points	*Criteria fullfilled*	*Not Available/*Not Applicable*	*Partially available*
General Information	Name/Address of applicant, description of the method, designation and specification of the GMM…		Title/scope of the project, conditions for placing the product on the market
Characterisation of the parental and derived organism	Characterisation of the parental organism and degree of relatedness, scientific name and taxonomy, phenotypic and genetic markers, identification and detection techniques, natural habitat of the parent organism, capability of the parent organisms to exchange genes, pathogenicity, safety for humans and animals, ecological and physiological traits, history of use of the parental organisms (QPS status)…		Genomic similarities of parental and derived organism
Characterisation of the GMM	Description of the genetic modification, purpose of the modification, stability	Origin of the inserted sequences*, description of the donor organism*, description of used synthetic DNA and nucleotide sequences, vectors*, genetic map of donor DNA*, rate and level of expressed genetic material and proteins*	Stability of the GMM
Information relating to the product	Production process, description, designation, intended use, toxicology, allergenicity (proteins, adjuvants)…		Composition, physical and technological properties, exposure , nutritional assessment, environmental impact

7. *Akkermansia muciniphila* as an Example of a Possible Novel Candidate in the Future

This chapter describes an attempt to consider the significance of *Akkermansia muciniphila* as an example of a novel probiotic candidate in the future, which failed to be detected and cultivated for an extended period of time (Derrien et al. 2004; 2008). Approximately greater than one percent of all faecal bacteria is able to degrade mucin, whereas *A. muciniphila* seems to be the first detected intestinal member of the phylum Verrucomicrobia (Derrien et al. 2010; Miller and Hoskins 1981).

7.1. Characteristics and Identity of the Organism

Akkermansia spp., a relatively newly detected bacterium, is widely distributed in animals and human intestinal tracts. It is presumed that *A. muciniphila* can be detected in up to 75% of the human population as well in breast milk (Collado et al. 2007; 2011). Akkermansia-like 16S rDNA sequences have also been analysed in domestic or wild mammals and vertebrates belonging to following orders: primates, rodentia, suidae, carnivora, sirenia, diprotontia, proboscidaea, ruminantia, lagomorpha and perissodactyla (Belzer and de Vos 2012). Therefore, it is apparent that the mucin degrader is highly adaptable and able to live in different anatomical varients (simple, foregut, hindgut) and under different conditions (diets, mucin types) (Ouwerkerk et al. 2013). The widespread occurrence, even in animals such as fish and Burmese pythons, emphasizes its evolutionary importance for the microbial ecosystem and points to mutualistic symbiosis of *Akkermansia* spp. with its host (Belzer and de Vos 2012; Costello et al. 2010; Derrien et al. 2010; Hildebrand et al. 2012).

The detection of the new genus *Akkermansia* ssp. occurred by investigating the diversity of mucin-degrading bacteria in the human intestine in 2004 (Derrien et al. 2004a; Straininfo 2014). Bacteria in human faeces samples were enriched and analysed by "denaturing gradient gel electrophoresis of PCR amplified 16S rRNA gene sequences" (Derrien et al. 2004a, pp. 470). By adding mucin to the growth medium, strain MUC^T (also known as ATCC BAA-835 or CIP 107961) could be cultivated. The strain belongs to a new species named *A. muciniphila* (Derrien et al. 2004a). It represents an abundant mucin degrading bacterium in the human intestine by colonizing the physically protective mucus layer by using the glycoprotein mucin as its direct source of carbon and nitrogen

(Derrien et al. 2004a; b; 2008). Table 15 summarizes the given characteristics of the mucin degrader.

Table 15: Description of the strain Akkermansia muciniphila (Derrien et al. 2004a; Straininfo 2014)

Cell morphology	Oval-shaped
Cell size (mm)	0.6-1.0 depending on the medium
Tolerance of oxygen	NO
Range of Tempearture (°C)	20-40
Optimum Tempearture	37
Growth on	No growth on *glucose, cellobiose, lactose, galactose, xylose, frucose, rhamnose, maltose, succinate, acetate, fumarate, butyrate, lactate, casitone (0.5 %), Casaminoacids (0.5 %), tryptone (0.5 %), peptone (0.5 %), yeast extract (0.5 %), proline, glycine, aspartate, serine, threonine, glutamate, alanine, N-acetylglucosamine or N-acetylgalactosamine* after 4 weeks incubation
Mucin degrading	Positive
DNA G+C content (Mol%)	47.6* 55.8 (van Passel et al. 2011)
Range of pH	5.5-8
Optimum pH	6.5
Doubling Time	1.5h in mucin medium
Flagella	Negative
Spores	Negative
***Determined by HPLC	

The oval shaped, gram negative, mucin-degrading bacterium *A. muciniphila* is strictly anaerobic, able to grow without vitamins and is an obligate chemoorganotroph. Surprisingly, the strain is capable of synthesizing all 20 proteinogenic alpha-amino acids, cofactors and vitamins (van Passel et al. 2011). The bacterial cells are non-motile but are covered with filaments when applied to mucin medium (Derrien et al. 2004a;b; van Passel et al. 2011). Gastric mucin enriched media for proper growth is required. However, the bacterium also grows on Columbia and BHI media but not with the same capacity as compared to mucin medium. As a result of mucin fermentation, the strain produces acetone, propionate ethanol, and sulphate ions. The strain occurs singly in chains or forms aggregates depending on which medium is used. *A. muciniphila* utilizes mucin as carbon,

energy and nitrogen-sources and therefore gains a competitive benefit during nutrient deficiency and under harsh conditions (Derrien et al. 2004b; Sonoyama et al. 2009). The mucin degrading enzyme complex is composed by α/β-D-galactosidase, α-L fucosidase, α/β-N-acetylgalactosaminidase, β-Nacetylglucosaminidase, neuraminidase, and sulfatase. Each single enzyme is necessary for the degradation of mucin and only several bacteria besides *A. muciniphila* are capable to do so (Derrien 2007; Derrien et al. 2010).

A. muciniphila benefits from the mucus layer, achieving a higher survival rate under harsh intestinal conditions and a higher abundance seems to be related to the body weight in mice and humans (Everard et al., 2011; Derrien et al. 2010; Karlsson et al., 2012¸ Santacruz et al., 2010; Teixeira et al. 2013). Additionally, due to its association with several intestinal disorders and its link to the protective mucus layer, *A. muciniphila* is believed to have a great potential as a biomarker for healthy individuals and treatment for several diseases (Berry and Reinisch 2013; Png et al. 2010; Swidsinski et al. 2011). Studies suggest that the mucin degrader is capable of modulating energy homoeostasis, glucose metabolism, obesity related inflammation and Diabetes mellitus Type 2. However, little is known to date about its functionality (Everard et al. 2012; Cani et al. 2014; Png et al. 2010; Shin et al. 2014; Swidsinski et al. 2011; van Passel et al. 2011). The correlation of *A. muciniphila* levels with different issues and diseases is summarized in Table 16.

Table 16: Role of the Akkermansia muciniphila level related to several issues and diseases

	Decreased	**Increased**
Level of *Akkermasnia muciniphila* in the host	Diabetes Mellitus Type I (Hansen et al. 2012)	Colorectal Cancer (Weir et al. 2013)
	Autism (Wang et al. 2011)	In obese pregnant women (Collado et al. 2012)
	Obesity and Overweight (Everard et al. 2013; Karlsson et al. 2012; Santacruz et al. 2010)	Fasting (Sonoyama et al. 2009)
	Inflammatory bowel diseases (Png et al. 2012; VigsnÆs et al. 2012)	
	Appendicitis (Swidsinski et al. 2011)	
	Atopic diseases (Candela et al. 2012)	

In vivo studies on Syrian hamsters or mice revealed not only a competitive advantage, but also an encouraged growth of *A. muciniphila* during starvation or by adding polyphenole, polyamines and prebiotics (oligofructose, inulin and arabinoxylans) (Everard et al. 2011; Gómez-Gallego et al. 2012; Kemperman et al. 2013; Sonoyama et al. 2009; van Abbeele et al. 2012). Inulin, arabinoxylans and others may increase mucin production and consequently increases the number of *A. muciniphila* and its beneficial metabolites (e.g. short-chain fatty acids) in germ-free mice (Derrien et al. 2004b; van Abbeele et al. 2012).

Derrien et al. (2008) proved with the help of a 16 rRNA-targeted probe and fluorescent in situ hybridization (FISH) linked with flow cytometry that *A. muciniphila* is common representative of the human microbiota in healthy adults as well babies, representing 1-4% of the intestinal microbiota (Derrien et al. 2008; Collado et al. 2007). It is interesting to consider the stable level of *A. muciniphila* in infants, which is already very close to that in healthy adults. Collado et al. (2007) confirmed these findings through a real-time PCR by targeting the 16 sRNA gene of *A. muciniphila.* The study concluded that a nearly constant level is reached by the age of one (10^8 cells/g feces) which declines again in the elderly (Collado et al. 2007).

Classification and Taxonomy

The most used method to characterize and identify microorganisms in the environment and in the human intestine, is real-time sequencing of the 16S rRNA genes. However, 16S rRNA genes are often very similar for multiple organisms, which poses an obvious problem. Therefore, newly introduced high-throughput approaches by using molecular markers have become rapid, low-cost technology for characterizing the microbial diversity. However, precise identification at the strain or species level remains difficult (Tu et al. 2014).

The species *A. muciniphila* belongs to the genus *Akkermansia* and is related to the deeply rooted order Verrucomicrobium and its genera *Prosthecobacter* and *Verrucomicrobium* (Derrien et al. 2004a;b; Hedlund et al. 1997). The most closely related bacterium with 92% is *Verucomicrobium spinosum* (Derrien et al. 2004a;b). Verrucomicrobia as a relatively new phylum, harbouring mainly clones and only a few bacteria are cultivatable (Hedlund et al. 1997). From the current point of view, the phylum is composed of five subphyla. The genera *Prosthecobacter* and *Verrucomicrobium* are

part of subphylum 1 (Hugenholtz et al. 1998). The first member of the phylum which has been isolated from the human intestine is named *Victivallis vadensis,* followed by *A. muciniphila* in 2004 *(*Staininfo 2014*;* Zoetendal et al. 2003). Strain Muc^T shares some chracteristics with its relatives, *Prosthecobacter* and *Verrucomicrobium*, but is unique in its anaerobic behaviour and oval shape (Derrien et al. 2004 2004a;b). Therefore it is assumed that *A. muciniphila* with its species Muc^T, forms its own genus of subphylum 1 of the Verrucomicrobia. Table 17 shows the taxonomic classification of *A. muciniphila*.

The complete genomic sequence and the entire 16 rRNA gene of strain Muc^T have been already determined. The 2,176 genes are condensed to one circular chromosome with an G+C content of approximately 47.6 - 55.8% (Derrien et al. 2004a;b; 2010; van Passel et al. 2011). Although *A. muciniphila* possesses a relatively small genome, it seems to be a specialist in mucin degradation in comparison to e.g. *Bacteriodes* spp. (Derrien et al. 2010). Approximately 60 genes are involved in mucin degradation with close relation to the genomes of *Bacteriodetes* and *Acidobacteria* species (Collado et al. 2007; Kamneva et al. 2012).

High throughput approaches such as metagenomics, allow capturing a wide variety of species by 16S rRNA sequences for an accurate quantification of the microbiota diversity in different individuals (Gill et al. 2006). In case of *A. muciniphila,* as a unique enteric representative of *Verrucomicrobia*, genomic data can easily be determined (Belzer and de Vos 2012). The data available suggest that several different species and strains of the genus *Akkermansia* can live simultaneously in the human gut.

Genes which are only present in *A. muciniphila* in comparison to other verrucomicrobial genomes, are linked to mucin degradation (11% of proteins might be involved), carbohydrate transport and metabolism, cell envelope biogenesis and outer membrane function (van Passel et al. 2011).

Table 17: Taxonomic classification of Akkermansia muciniphila (LPSN n.d.)

PHYLUM	VERRUCOMICROBIA
CLASS	Verrucomicrobiae
ORDER	Verrucomicrobiales
FAMILY	*Akkermansiaceae*
GENUS I	*Akkermansia*
SPECIES	*Akkermansia muciniphila*
STRAIN(S)	*ATCC BAA-835^T= CIP 107961^T= Muc ^{T} (and others)*

7.2. Safety Status of *Akkermansia muciniphila*

Chapter 4.2 of this work deals with the QPS system suitable for organisms with a long and safe history of use. In terms of the relatively newly identified and characterized bacterium *A. muciniphila*, it might be questionable if the QPS status could apply due to a lack of required data and other information. Thus, complete safety assessment is required.

Body of Knowledge

A. muciniphila, the first time described in scientific literature in 2004, the year of origin is dated back to 2002 according the biological resource centre ATCC with M. Derrien as its depositor (ATCC 2014; Derrien et al. 2004a).

Although the species has not been commercially sold as a probiotic since its detection, it is an abundant inhabitant in human and other animal guts and is well-studied in several human trials (Belzer and de Vos 2012; Costello et al. 2010; Derrien et al. 2010; Karlsson et al. 2012; Ouwerkerk et al. 2013; Png et al. 2010; Santacruz et al. 2010; Swidsinski et al. 2011; Zhang et al. 2009). *A. muciniphila* is not involved in food production or industry to date, but has potential in treating obesity, diabetes mellitus type 2, gastrointestinal diseases and other metabolic dysfunctions (Everard et al. 2012; Karlsson et al. 2012; Png et al. 2010; Santacruz et al. 2010; Zhang et al. 2009). However, viable cells of *A. municihila* have only been deliberately administered in animal models so far (Everard et al. 2012; Kang et al. 2013; Shin et al. 2014). No clinical trial exists yet where viable cells of *Akkermansia* spp. were administered to humans with the purpose of studying the effect and impact on the human organism. Available study approaches focus for instance on the impact of administered probiotics, plant extracts and medications on the microbiota and therefore on *Akkermansia* spp. itself (Grześkowiak et al. 2012; Shin et al. 2014, Wang et

al. 2014). Furthermore, not a single health claim proposal has been submitted over the years (European Commission 2014).

- Scientific literature

To assess the body of knowledge, a literature research under the same terms as previously described in Chapter 5.2, was performed. Both searches came to similar outcomes at the date of the search (28. June 2014). The results are displayed in Table 18 below. Only articles and works obtained by Scopus, Pubmed and reltated databases were evaluated and reported in this work. As the search results indicate, the available scientific literature of *A. muciniphila* is limited but sufficient for a cursory examination.

Table 18: Results of all searches regarding Akkermansia spp. in different databases (28. June 2014)

Database	**Search term**	**Hits (in article, title, abstract, keywords)**	**Hits (in title)**
Scopus	*Akkermansia*	81	13
	Akkermansia muciniphila	2	1
Pubmed	*Akkermansia*	75	
	Akkermansia muciniphila	46	
Google Scholar	*Akkermansia*	955	
	Akkermansia muciniphila	12	

- Pathogenicity

In general, *A. muciniphila* has not been clearly correlated to any disease or sign of pathogenicity so far (Derrien et al. 2010). However, doubts concerning its safety were expressed in some scientific works.

Firstly, adhesion to the mucous layer and the extended mucin degradation could also be involved in initial pathogenic behaviour (Derrien et al. 2004b; 2010; Donohue and Salminen 1996; Tuomola et al. 2001). Unlike pathogens, *A. municihila* as a mucin-degrader, colonizes mainly the outer mucus layer and not the inner one, which retains a final physical barrier. Bacterial translocation has not been observed or reported in scientific literature.

It should not be forgotten that mucin degradation is a normal and stable process in the gastrointestinal tract of healthy individuals (Derrien 2004b; 2007). It is also assumed that the A. muciniphila might be an important microorganism for maintaining the microbial

balance in the hosts' intestine by converting mucin to beneficial side products (Derrien et al. 2004b).

A study carried out by Kang et al. (2013) gives little evidence that *A. muciniphila* could be detrimental in case of disturbed barriers in mice with dextran sulfate sodium-induced colitis. The team suggested that dextran sulphate sodium reduces the thickness of the mucus layer and therefore makes it more susceptible to microorganisms. On the contrary, administration of extracellular vesicles produced by *A. muciniphila,* generated positive outcomes in colitis mice as decreased body weight loss, increased colon length, and lower levels of inflammation indicators (Kang et al. 2013). To related outcomes came a study testing the effects of *A. muciniphila* on *S.typhimurium*-infected mice. The results showed that an administered dose of 10^8 cfu/day together with the pathogen, increased the grade of inflammation. No evidence was given that *A. muciniphila* hosts pathogenic characteristics alone. However, it cannot be excluded that the commensal strain converts itself to a pathobiont in the presence of other bacteria and in susceptible individuals (Chow and Mazmanian 2010; Ganesh et al. 2013). This is not a new discovery, as other commensal bacteria and probiotics can be harmful under given circumstances (FAO/WHO 2002).

There is also evidence that a disturbed microbial balance is associated with colorectal cancer, with *Akkermansia* genus potentially as a contributor. Tumour-affected germfree mice showed an increased level of *Akkermansia* ssp. among other bacteria. After a treatment with antibiotics, the size and number of tumours diminished dramatically, indicating that an imbalanced microbiota could work synergistically with other tumour inducing factors. Additionally, the transfer of the disturbed microbiota into healthy objects, enhanced tumour growth in number and size (Zackular et al. 2013).

Stool samples of colorectal cancer patients also showed a higher level of *A. muciniphila* compared to those of healthy ones, while butyrate-producing bacteria were decreased. *A. muciniphila*, with a rate of 3.54% in healthy subjects was 4-fold lower than in those suffering from colorectal cancer (12.8%) (Weir et al. 2013). Those findings indicate a correlation of several factors together with *A. muciniphila,* which lead to adverse effects and health consequences.

A. *muciniphila* as a gram-negative bacterium habours lipopolysaccharides but no correlation to endotoxemia could be investigated thus far. Surprisingly, the bacterium

even lowered levels of endotoxins in correlation with high fat diet in mice (Everard et al. 2012). The mucin degrader is also known to modulate inflammation and the immune system through signals such as TNF TNF-α, IFN-γ, IL10 and IL4 (Collado et al. 2012; Derrien et al. 2011). There are tendencies that lower levels of anti-inflammatory IL10 and IL4 as well as tendencies that increase levels of the pro-inflammatory TNF- α and IFN- γ that are assosciated with higher amounts of *Akkermansia* ssp. in breast milk (Collado et al. 2012). Increased levels of the bacterium have also been associated with allergic diarrhea. The author explains this hypothesis by higher mucin degradation and subsequently enhanced allergenic protein uptake in the gastrointestinal tract (Sonoyama et al. 2010).

An anti-inflammatory and protective role in the gut is correlated with *A. muciniphil*a in other scientific reports (Candela et al. 2012; Png et al. 2012). To manifest these findings further investigations is required.

From a genetic point of view, *A. municiphla* possesses capsule building two genes. These are not believed to play a role in pathogenic behaviour, as they are necessary to protect against desiccation on the faecal-oral route (Ophir and Gutnik 1994; van Passel et al. 2011). Aside from these, deliberated *A. muciniphila* colonization in germ-free mice resulted in no adverse effects or up-regulation of any pro-inflammatory genes (Derrien et al. 2011).

High abundance in animals and humans enhances the species' non-pathogenic nature (Belzer and de Vos 2012; Gomez-Gallego et al. 2012). Furthermore, a decreased number of *A. muciniphila* and not of other mucolytic bacteria is associated with inflammatory bowel disease, especially Crohn's disease and ulcerative colitis. Due to its predominant attachment to the mucus layer, it can be assumed that the bacterium inhibits the colonization of other pathogens. This is likely to be disturbed if not enough substrate is available for *A. muciniphila*, resulting in a microbiota imbalance (Belzer et al. 2012).

- Antibiotic resistance and gene transfer

A study conducted in humans revealed that the Muc^T strain is susceptible to imipenem, piperacillin/tazobactam and doxycycline. This is reasonable due to its content of peptidoglycan (Derrien et al. 2008; Dubourg et al. 2013). Resistance behaviour against

vancomycin, metronidazole, and penicillin G were observed. The detected MIC levels of strain Muc^T are displayed in Table 19 (Dubourg et al. 2013).

Treatments with a broad-spectrum antibiotics, metformin and vancomycin alone did not necessarily reduce the number of *A. muciniphila*. On the contrary, the treatments increased the abundance of the bacterium in the intestine of mice and humans (Dubourg et al. 2013; Hansen et al. 2013; Shin et al. 2014). A comprehensive gene analysis of *A. muciniphila* exposed beta-lactamase genes (classes C and A) in the genome (Amuc_0106 and Amuc_0183), in addition to a 5-nitroimidazole antibiotic resistance protein (Amuc_1953) and a gene for antibiotic biosynthesis monooxygenase (Amuc_1805, PFAM PF03992) (van Passel et al. 2012). This type of monooxygenase has also been detected in the pathogen *B. subtilis* and requires further investigations (Park et al. 2012).

The enzyme beta-lactamase, which has also been detected in *Enterobacteriacea* and *Pseudomonas aerugi*nosa, is effective against diverse beta-lactam antibiotics such as ceftazidime, ceftriaxone, cefotaxime and oxyiminomonobactam. It is cited as a catalyst for spreading antibiotic resistance. Beta-lactamase of class A is especially associated with transmitted resistances among different species (Ghafourian et al. 2015). Resistances against the broadly used antibiotic 5-nitroimidazole are a common feature of many anaerobic bacteria, such as the non-pathogenic *Bacteroides fragilis* group (Fang et al. 2002).

Due to its close relation and possible interaction with other species (e.g. *Bacteroidetes*), horizontal gene transfer of antibiotic resistance genes cannot be excluded (Kamneva et al. 2012). Knowledge about genetic diversity, taxonomy and phylogenetic relatives is helpful in excluding horizontal gene transfer in terms of the abundant mucin degrader *A. muciniphila* (Salyers et al. 2004).

Table 19: Detected MIC levels of Akkermansia muciniphila strain Muc^T (Dubourg et al. 2013)

Strain	**MIC (µg/ml)**				
	Imipenem, Piperacillin/ Tazobactam	Doxycycline	Vancomycin	Metronidazole	Penicillin G
Muc^T	0.7	0.38	>64	>64	2.8

- Intended Use

The abundant commensal bacterium *A. muciniphila* has not been deliberately administered to humans. However, the commensal inhabitant shows great potential for future use. The species is highly recommended as a biomarker for intestinal diversity and the development of microorganism, as different populations show divergent percentages of this species (Belzer and De Vos 2012; Grześkowiak et al. 2012). Especially for common diseases such as inflammatory bowel conditions, adipose tissue related inflammations or non-alcoholic fatty liver diseases, tendencies for clinically relevant results are given after deliberate administration of *A. muciniphila* (Miller and Hoskins 2014; Png et al., 2010; Shin et al. 2014). Few scientific works recommend the bacterium for restoring an imbalanced microbiota or using it for prevention of adipose related metabolic diseases (Everard et al. 2013; Png et al., 2010; Santacruzet al., 2010). This was shown in mice, where the mucin degrader was able to reverse the adverse effects induced by a high-fat diet, such as endotoxemia, fasting hyperglycemia and insulin resistance (Everard et al. 2013).

A. muciniphila may also play a protective role in infants if administered orally by reducing the occurrence of autoimmune diseases (Hansen et al. 2012). Due to the detection of altered *A. muciniphila* numbers in association with rare diseases such as autism, it is important to reveal the complete relationship in relation to health and disease in order to implement the bacterium as a probiotic (Wang et al. 2011).

A. muciniphila shows potential as a probiotic by surviving the gastrointestinal passage. Its ability to adhere to the mucus layer has been suggested as a favourable probiotic characteristic and beneficial effects are only observed when the bacterium is administered in viable form (Everard et al, 2013; Derrien et al. 2010). To date, it is not scientifically proven if *A. muciniphila* alone is capable of modulating human parameters (Axling et al. 2012). Further studies are required to observe the detailed interaction pathway of *A. muciniphila* with regard to other bacteria and the host, as well its involvement in pathogeneses (Collado et al. 2007; Everard et al. 2013).

7.3. *Akkermansia muciniphila* as a Novel Probiotic?

A. muciniphila, as a newly discovered commensal bacterium, would be the first bacterium that is regulated by novel food regulation (EC) No 258/97. The bacterium, or a product containing it, has not been introduced to market to a "significant degree" before 15 May 1997.

Hence, market introduction of a probiotic product containing *A. muciniphila* requires a full safety assessment as a prerequisite to its approval. If a simplified procedure due to sustainable equivalence is appropriate, this would relate to another topic of discussion (European Commission 1997c). Along with the Commission Recommendation 97/618/EC, *A. muciniphila* would fall into category 2.2.: "the source of the NF has no history of food use in the Community". Table 20 illustrates available information which would be required for a complete approval of a product containing this novel microorganism (European Commission 1997a).

The most important point to establish would be if a microorganism with no history of safe use harms consumers' health and nutritional supply, in any way, when ingested orally and regularly. Randomised double-blind placebo controlled clinical trials and data about the final product and its formulation, its chemical, nutritional and technical structure and the applied process are obligatory for clarification. Knowledge about the cultivation and analysis of *A. muciniphila*, as described in the work of Derrien et al. (2004a), is an important step towards product development.

It might be beneficial to recommend a dose of the bacterium to guarantee health effects or avoid adverse effects. Due to the fact that *A. muciniphila* is an abundant commensal bacterium in the human intestine, a dosage of at least 10^8 cfu/day (depending on age and body weight of the subject) would be reasonable (Verna and Lucak 2010). The bacterium itself is taxonomically well-described and provides almost all favourable probiotic characteristics, such as adhesion to luminal mucosa and colonization of the gut. Although direct health effects are not documented as as of yet since the history of the bacterium is relatively short (Derrien et al. 2004a; b).

Table 20: Missing and already available information, which would be required and necessary for a complete approval of a product containing the novel bacterium Akkermansia muciniphila according the Commission Recommendation 97/618/EC (European Commission 1997a)

	Available	**Not Available/*Not Applicable**	**Partially available**
Specification of the NF	Species and Taxon	Chemical composition, nutritional properties of the product *	Toxicological information
Production process	The bacterium must be alive	Process, conditions, technical details *	Culturing methods
History of use of the source of the NF	Commensal bacterium and the source organism is categorized	No counterpart available, nor information about the past and the present usage	Source organism is not harmful for humans
Anticipated human intake/extent	Commensal bacterium	No intake patterns are known	
Nutritional information		No nutritional assessment in human models	Nutritional assessment in animal models
Toxicological information	Commensal bacterium	No traditional counterpart, no toxicological assessment in humans	Animal feeding models
Microbiologic information	Commensal bacterium		Pathogenic and toxicological nature, analysis of its metabolites
Allergenic potential	A direct association of bacterial derived proteins with allergy is unlikely, but contribution is possible (Sonoyama et al. 2010)		
Genetic engineering	No genetic modification so far		

*No product containing *A. muciniphila* has been placed on the market so far

A. muciniphila is known to be a commensal and abundant gut bacterium. Although it has no history of safe use as a commercially traded product, it has always been part of human and animal lives (Derrien et al. 2004a). The bacterium is associated with several health conditions, but is generally not believed to act as their cause (see Chapter 8.2.). According to Commission Recommendation 97/618/EC, substantial equivalence in terms of *A. muciniphila* may be not suitable. This simplified procedure is limited to microorganisms in food production, which have a traditional use in food and an appropriate counterpart (European Commission 1997a).

Of interest to future scientific works is if products with viable probiotics, such as *L. reuteri*, would be equivalent to a product containing the commensal inhabitant *A. muciniphila*. It can be assumed that *A. muciniphila*, as a natural inhabitant of the humans gut, provides the same safety status as commercially used probiotic bacteria (Casas and Dobrogosz 2000; Derrien et al. 2004a;b**)**. To prove this theory, clinical safety studies and further animal feeding models are necessary.

In terms of the European QPS System, no gram-negative bacterium except *Gluconobacter oxydans* has been approved (EFSA 2013). It is unlikely that *A. muciniphila*, which lacks safe history of use, will be approved as a QPS organism in the near future. However, many criteria that are required by the QPS system are already or partially fulfilled, such as taxonomic identity and exclusion of pathogenicity. This was discussed in Chapter 7.2. . Although the species brings along no history of use, the complete characterization of the strain might be sufficient to balance lacking knowledge in this respect (Derrien et al. 2004a; van Passel et al. 2011).

Data concerning exposure and ecological impact might be fulfilled with the knowledge that *A. muciniphila* is an abundant colonizer in humans, animals and infants (Belzer and de Vos 2012; Collado et al. 2007; Costello et al. 2010; Derrien et al. 2010; Hildebrand et al. 2012). Naturally, exposure to approximately 1–2 kg of intestinal bacteria does not equal deliberate ingestion of smaller amounts of "potentially probiotic bacteria", but as of yet, no safety concerns have been identified (Verna and Lucak 2010). If a bacterium is part of the natural intestinal microbiota, it may be supposed that this microorganism will not harm the host even when orally administered in high doses. In terms of susceptible individuals, administration of *A. muciniphila* should be treated with caution to avoid complications (Antony et al. 1996; Antony 2000; Bernardeau et al. 2008).

All this information, coupled by scientifically proven beneficial physiological effects, will be necessary to permit subsequent health claims for promoting the probiotic product.

This has been proven to be very difficult in terms of probiotics due to:

1.) Lack of established health effects
2.) The prohibition of medical claims for food.
3.) The remaining uncertainty of beneficial physiological effects in the healthy population hinders investors to initiate the much needed clinical trials for potentially effective probiotic product (European Commission 2006; Katan 2012).

7.4. Outlook

Based on the example of *A. muciniphila* we see that the repertoire of proposed new probiotic candidates has not been exhausted (Derrien et al. 2004a). Owing to the development and improvement of "high-throuput and next generation sequencing techniques", the potential of new probiotic bacterial species can be more easily elucidated (Neef and Sanz 2013).

A. muciniphila as a mucin degrader is an appropriate candidate among other bacteria, such as *Clostridum butyricum, Clostridia c*lusters IV, XIVa (*Eubacterium rectale*) and XVIII, as well *Bacteroides uniformis* (Duncan and Flint 2008; Neef and Sanz 2013). *Faecalibacterium prausnitzii* and members of the family *Lachnospiraceae*, such as *Roseburia* ssp., *Coprococcus* ssp., and *Eubacterium rectale* have all been depleted in association with inflammatory bowel diseases. Therefore, they possess potential as future alternative treatments (Berry and Reinisch 2013; Sokol et al. 2008). Even among well-studied species, new potential strains are regularly discovered. Such is the case with *Lactobacillus acidophilus* strain 36YL (Nami et al. 2014). Strains of *Veillonella*, in combination with *Propionibacterium* spp., may be beneficial in infant formula. In this context, it should be taken into account that combinations of potential bacteria may enhance or decrease the desired effects (Chassard et al. 2014; Collado et al. 2007).

All these candidates, especially *F. prausnitzii* and *A. muciniphila*, possess some interesting potential in preventing and treating specific diseases and thus might be termed as "second generation probiotics" (Underwood 2014). In comparison to *L. reuteri* strain ATTC 55730 which is already commercially available on the market as a probiotic formula, *A. muciniphila* is still in its infancy and requires the cooperation of researchers, industry, and regulatory bodies. The first obstacle which must be overcome is the design of a safe formulation of *A. muciniphila,* followed by clinical trials with dose escalation (Underwood 2014).

At present, obesity and related non-communicable diseases are global challenges, manipulation of the intestinal microbiota would be a comfortable and welcomed solution to enhance weight loss. For instance, *Lactobacillus gasseri* showed that a market exists for probiotics associated with obesity (Miyoshi et al. 2014).

Therefore, *A. muciniphila* as a novel probiotic would have potential to achieve success as a commercial product. Although it seems likely that the lack of clinical studies phase 1 to 3 prolongs the launch of novel products (see Chapter 2.3) (Everard et al. 2011; Karlsson et al. 2012; Santacruz et al. 2010). In the future, scientific work should focus on the function and importance of *Akkermansia* spp. in humans to facilitate the launch of novel probiotic formulations. *A. muciniphila* has potential to reduce private and public health costs by dealing with different conditions and by acting as disease prevention (Indio et al. 2014). Therefore, it would be of importance to conduct human intervention studies to identify the role of Akkermansia in intestinal mucosa and to monitor any side effects during its consumption.

Bacterial strains, which are believed to harbour potential antibiotic resistances, do not have to be necessarily excluded by the QPS system, as in probiotics non-transferable resistances to one or few antibiotics could be regarded as harmless. The risk of resistance transfer of human commensals to pathogens might be low due to the rare occurrence of mobile resistances in probiotic strains. Theoretically, controlled antibiotic resistances could be beneficial due to higher survival rate and by diminishing gastrointestinal side effects during co-administration with antibiotics (Courvalin 2006).

The plasmid-cured strain *L. reuteri* DSM 17938 acts as an example for potential future opportunities. The applied plasmid-curing technique appears to be safe, since no induced side effects (such as mutations) have been identified. Enhanced techniques regarding plasmid elimination facilitated by heterocyclic compounds or physical methods could inhibit the spreading of horizontally transferred antibiotic resistance plasmids worldwide. Moreover, deliberate plasmid eliminations could reveal new opportunities in biotechnology research and potentially decrease bacterial virulence behaviour (Spengler et al. 2006).

A few points with respect to the European QPS system and other regulatory approaches are topics of concern as well. Firstly, a possible "sectorisation" of the market should be addressed, since large international companies could gain a competitive advantage over smaller ones with limited resources. However, through the consistency of the application scheme, no unequal benefits among notifiers should be warranted.

Secondly, molecular tools and other techniques differ in the scope of applications, viability and liability. Consequently, the measurement techniques have an impact on the

"identity" and "body of knowledge". Therefore, newly developed and standardized techniques should be considered by the panel and notifiers. Lastly, the acquired properties of genetically modified microorganisms are not covered by the generic safety assessment and should be discussed prospectively.

For an operational generic safety assessment, other points additional to the four main pillars might be relevant as well. Besides "traditional use", the daily exposure should not be underestimated, as several bacteria such as *A. muciniphila* are widespread in our environment or intestines and would open doors for novel probiotic applications. Additionally, it is not yet clear at what point exactly the novel status ends and the bacterium gains conventional reputation.

In terms of improving consumer faith, it seems important not to raise extra concerns among the population. Citizens have already been sensitized about "bad bugs". Therefore, the communication to the public remains an important topic. People should not believe that the EFSA does not deal anymore with microbial risks as it did before the QPS introduction (EFSA 2005a).

In 2012, the QPS system has gained legal status with regard to food enzymes, according to Regulation (EC) No 562/212. The regulation specifies that any application of microorganisms with a QPS status used for enzyme production must not include "toxicological data" (European Commission 2012). This exception could be the leverage point for further QPS integrations into European legislation. However, the QPS system remains a pure safety assessment tool without management character although it is meant to be of help to all EFSA expert activities and scientific panels (EFSA 2005a).

The newly proposed version of the NFR aims a "centralised harmonisation", meaning no simplified application processes by "substantial equivalence" anymore. The responsibility of the initial assessment will be transferred to the Commission and will no longer lie to the member states. To date, "substantial equivalence" is not officially applied to microorganisms in Europe, but would be an interesting and welcomed approach for newly detected commensal bacteria in the future. However, the two related strains *L. reuteri* ATCC 55730 and DSM 17938 act as an example for "substantial equivalence" which has not yet been questioned by the EFSA or other regulatory agencies.

Figures

Tables

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