Aus dem Institut für Tierernährung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Selection, Preparation and Application of Host-derived Probiotic Lactobacilli from Chicken and their Synergistic Effects with Phytobiotics on Broilers

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der Freien Universität Berlin

vorgelegt von
Hao Ren
Tierarzt aus Huhhot, China

Berlin 2020
Journal-Nr.: 4193

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Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Jürgen Zentek
Zweiter Gutachter:	Univ.-Prof. Dr. Uwe Rösler
Dritter Gutachter:	Univ.-Prof. Dr. Marcus Fulde

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CHAPTER 1: GENERAL INTRODUCTION

In the past decades, the consumption of poultry meat has increased considerably to supply a cost-effective protein source to a constantly growing population worldwide (Figure 1) (Hannah and Max, 2019). An interesting statistic is that the feed consumption and time duration to promote the body weight of broiler chicken to 1.8 kg in the 1920s were over 20 kg feed in 112 d (Diarra and Malouin, 2014). However, commercial broilers nowadays achieve the same body weight only with an average consumption of 3.2 kg feed for approximately 35 d, which perfectly reflects how the poultry nutrition development improved the production efficiency of animals. The great improvement of feed conversion in the last century is attributed to the advances in animal breeding and nutrition. However, despite a better utilization of nutrients in poultry diets, in-feed growth promoters have played a fundamental role in the rapid improvement of performance. In the past, the most efficient and most often used growth promoters were antibiotic growth promoters (AGP), including virginiamycin, bacitracin, methylene disalicylate and other AGP, which have been used for more than 60 years (Castanon, 2007). However, antibiotic resistance has emerged as a new problem due to the long-term usage of AGP. Hence, the use or even abuse of antibiotics in livestock is criticized for increasing the presence of antibiotic resistant bacteria in animals (Economou and Gousia, 2015). What is worse is that the reservoirs of resistance in animals are able to transmit to humans via the food chain. Sweden was the first country to respond to AGP-driven resistance development by banning the usage of AGP in 1986. Many other countries joined to prohibit the supplementation of AGP in animal feed (Wierup, 2001; Millet and Maertens, 2011).

The public demand for substituting AGP in animal nutrition initiated a worldwide search for alternative feed additives that can replace AGP. In recent years, different types of feed additives were investigated to compensate the loss in performance when APG are absent. Probiotics and phytobiotics are two extensively studied alternatives and have shown great potential to improve animal performance, gut microbiota or the immune system (Chaucheyras-Durand and Durand, 2010; Amara and Shibl, 2015; Hussein and Selim, 2018; Abouelezz et al., 2019; Thirumal et al., 2019). However, proven probiotic strains were isolated from vast sources such as cheese, kimchi or soil, it is of question whether they will survive and colonize well in other different host species. The host specificity of probiotics is often neglected during selection of suitable strains. As reportedly, the host-specific origin may enhance the colonization and further function of probiotic bacteria in host intestine (Zmora et al., 2018). Nevertheless, host-specificity has not been regularly regarded as a selection criterion for determining the candidate strain in probiotic feed additive. On the other hand, as two frequently tested alternatives, probiotics are rarely used with phytobiotics as combination in feed additives. Many reports have demonstrated that combinations of probiotics with other feed

additives such as prebiotics or acidifiers have favorable effects, but only two studies employed the combination of probiotics and phytobiotics with each other (Erdoğan et al., 2010; M. Abdel-Raheem, 2016; Liu et al., 2018; Calik et al., 2019).

On basis of the facts above, the preparation and verification of poultry-specific feed additives will be of high interest in the field of animal nutrition. Therefore, this PhD thesis describes the isolation, selection, formulation and evaluation of probiotics and phytobiotics for poultry nutrition. The first part of the thesis was performed to establish a rapid and high-throughput screening method to select competent candidate probiotics for the further use. Over 7000 colonies from broiler digesta and excreta were subjected a 3-staged screening method, in which 2 final candidates were selected by *in vitro* and *ex vivo* criteria. Thereafter, the candidates were characterized by taxonomic identification, aggregative ability, and resilience against gastric stress as well as their antibiotic resistance profile.

For a better evaluation *in vivo*, two candidate *Lactobacillus* strains were optimized in terms of production parameters. The metabolic fingerprints of the strains were assessed with BIOLOG AN® plates to find substrates which may booster biomass production. The capacity to grow under aerobic condition to ease large-scale biomass production was also determined. Furthermore, to maintain the maximum viable cells upon storage, a protective medium consisting of skim milk, sucrose and trehalose was developed for each strain via optimization with the response surface method (RSM). The storage stability of formulated probiotics was evaluated under both room temperature and refrigeration.

The third part of the thesis focuses the *in vivo* effect of two probiotic feed additive, two phytobiotic additives and their corresponding combinations on young broiler chickens. The feed additives were supplemented into starter feed for 200 broiler chickens in 9 groups. After 14 d feeding, birds from each group were sacrificed. The modulation of their gut microbiota was analyzed with samples from crop and caecum by 16S rDNA analysis, qPCR and metabolome analysis. Additionally, the survival of an extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* in intestinal samples was determined *ex vivo* to estimate the inhibiting capacity of each feed additive or their combinations on this antibiotic resistant strain. Overall, the results of the feeding trial indicated that the synergistic effects beneficial modification of the intestinal microbiota and inhibition of the ESBL *E. coli* strain.

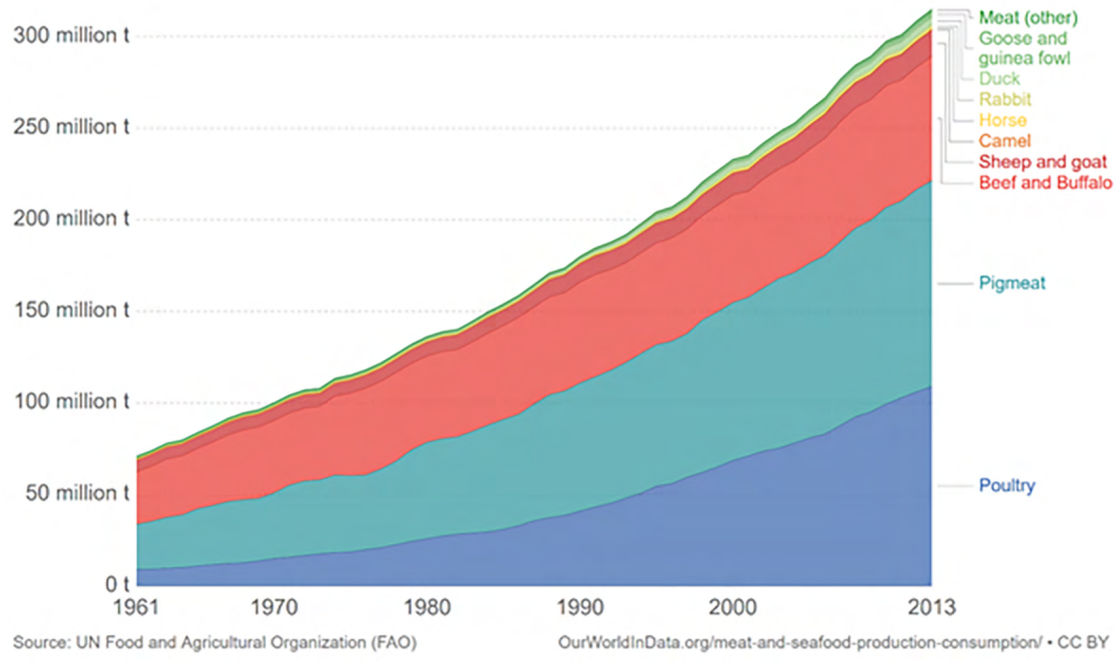

Figure 1: The worldwide meat production by livestock from 1961-2013

Data source: Food and Agriculture Organization (FAO)

CHAPTER 2: LITERATURE REVIEW

2.1 Poultry nutrition and feed additives

The global human population is predicted to reach 9.7 billion by year of 2050 (Hunter et al., 2017), and thus creates a high demand of animal protein source (Boland et al., 2013). As an eco-friendly animal protein source, poultry meat primarily contributes to the rapid expansion of meat consumption and future demands worldwide (FAO, 2018). Poultry species are characterized by higher feed conversion rate, as well as the higher cost-efficiency than other livestock (Wahyono and Utami, 2018). In poultry production, the major cost is feed (up to 70%), 95% of which is used to meet energy and protein requirements (Ravindran, 2013). The commonly used ingredients in poultry nutrition are shown in Table 1 (Ravindran, 2013). Accordingly, poultry feeds consist primarily of grains and oilseed meals (Rochell, 2018). Along with the development of modern poultry breeding, the related products (meat or eggs) are expected to enjoy faster and greater production during rearing. Feed additives are used in animal nutrition to improve the characteristics of feed and are extensively applied in animal industries on scale (Rychen and Toussaint, 2018), it includes five categories, namely zootechnical additives, sensory additives, nutritional additives, zootechnical additives and coccidiostats and histomonostats based on EU regulation (Regulation EC. No 1831/2003). Therefore, an essential goal of feed additives is to achieve rapid and sustainable animal production (Beski et al., 2015). Consequently, the formulation of poultry feed is encouraged to not only simply fulfil energy or amino acid demands, but also to maximize nutrient availability (Ravindran, 2005).

Animal feeds have a certain composition of nutrients and are designed to fulfill the requirements of animals at different life stages. However, there are substances that lower the availability of nutrients (Yacout, 2016). These substances are called anti-nutritional factors (ANFs). The ANFs are categorized into four chemical groups, namely protein ANFs, Glycosides ANFs, Phenol ANFs, and Miscellaneous ANFs (Sarwar Gilani et al., 2012). These substances can inhibit the nutrition utilization via modulating protein digestion, mineral utilization and anti-vitamin factors and miscellaneous (Francis et al., 2001).

To enhance performance and animal health for better production and financial results, growth promoters are chemical and biological substances which are added to livestock feed as additives (Fallah et al., 2013). Various types of feed additives, for instance antibiotics, probiotics, prebiotics, phytobiotics, synbiotics, enzymes or antioxidants, have been developed to maximize animal growth and to improve animal health. AGP have been broadly used as feed additives to ensure not only high resilience against infection but also improvement on growth performance for over 60 years (Dahiya et al., 2006; Castanon, 2007). Numerous studies endorse the positive impact of AGP on body weight (BW), BW gain, feed conversion

ratio (FCR) and egg production to poultry at subtherapeutic doses (Mehdi et al., 2018). Yet, the intensive use of AGP has contributed to the development and increase of antibiotic resistant bacteria, and pose a huge threat to both animal and human health (Aarestrup, 2015; Nelson et al., 2019). Thus, many countries and territories have responded by banning the use of AGP for the environmental and public health concern (Costa et al., 2017). Due to the absence of APGs in feed, growing interests focus on the development of alternative additives to substitute the growth promotion. The 'green' feed additives are characterized by high efficiency, high biosafety and low selection pressure during the feeding. A list of potential alternatives is summarized in Table 2. More details regarding probiotics and phytobiotics will be given in the following sections.

Table 1 Commonly-used feed ingredients for poultry dietary formulation (Ravindran, 2013)

Ingredient type	Majorly-used ingredients
Energy sources	cereals (maize, wheat or sorghum), cereal by-products, animal fats and vegetable oils
Plant protein sources	soybean meal
Animal protein sources	fishmeal, meat and bone meal
Mineral supplements	limestone, shell grit, dicalcium phosphate, defluorinated rock phosphate, bone meal, salt, sodium bicarbonate
Miscellaneous	vitamin premixes, methionine, lysine, threonine, non-nutritive feed additives (enzymes, antibiotics, etc.)

Table 2 Examples of alternative feed additives

	Example	Effect	References
Probiotics	*Lactobacillus* spp., *Bacillus* spp., *Enterococcus* spp. *Saccharomyces* spp.	FCR ↑, immune response ↑ microbiome maturation ↑ nutrient digestibility ↑ intestinal pH ↓ pathogenic bacteria ↓ BW gain ↑	Koc et al., 2010; Levkut et al., 2012; Gao et al., 2017; Reis et al., 2017
Phytobiotics	Carvacrol, thymol, cinnamic aldehyde, rosemary extracts	BW ↑ blood cell ↑, meat quality ↑ egg quality ↑	Alagawany et al., 2017; Reis et al., 2018
Prebiotics	Mannanoligosaccharides	BW gain ↑, feed intake	Iriyanti et al.,

	(MOS), fructooligosaccharides (FOS), Xylo-oligosaccharides (XOS), inulin	↑ villus height ↑, immune organs ↑	2018; Biswas et al., 2019; Rahimi et al., 2019
Synbiotics	*Lactobacillus* spp. + lactose, *Bacillus subtilis* + FOS	FCR ↑, BW ↑, diarrhea and mortality ↓, immuno-stimulation ↑	Vicente et al., 2007; Li et al., 2008; Awad et al., 2009
Acidifiers	formic acid, ascorbic acid, butyric acid, lactic acid and their salts	Nutrient digestibility ↑, specific antibody titer ↑, BW gain ↑, cumulative feed consumption ↓	Lohakare et al., 2005; Garcia et al., 2007; Panda et al., 2009; Hamid et al., 2011
Enzymes	Proteases, xylanases, β-mannanases	BW ↑, FCR↑, villus height ↑, crypt depth↓	Cowieson et al., 2006; Sardar, 2006; Mehri et al., 2010

2.2 General background of in-feed probiotics and phytobiotics

2.2.1 Probiotics

As defined by Food and Agriculture Organization/ World Health Organization (FAO/WHO), probiotics for human use are 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). In the field of animal nutrition, the concept of probiotics particularly targets the microorganisms that protect the animal against specific pathogenic bacteria or have beneficial effects on animal performance and health (Markowiak and Śliżewska, 2018). The benefits of probiotics have been highlighted and studied ever since Élie Metchnikoff firstly theorized that 'host friendly bacteria' were able to promote health in humans one century ago (Mackowiak, 2013). The concept was later improved by Ferdinand Vergin who introduced the term 'Probiotika' coming from the Greek terms "pro" and "bios" in the year 1954 (Ebner et al., 2014). After the first probiotic-based product was commercialized in 1905 as bacteria-fermented drink known as 'Yakult' (Amara and Shibl, 2015), which is still manufactured today, many applications were developed based on various bacterial species for diverse purposes (Kajander et al., 2010; Liu et al., 2014; Lan et al., 2016). For a long time the agricultural application of probiotics for farm animals did not receive as much attention as for human use, but it is gradually being regarded as a targeted additive with increasing interest (Martínez Cruz et al., 2012; Fox, 2015).

Although most existing probiotics are bacteria, other microorganisms like yeasts or filamentous fungi also belong to group of probiotic (Czerucka et al., 2007; Palma et al., 2015; Sugiharto et al., 2015). *Lactobacillus* and *Bifidobacterium* spp. are most commonly used probiotics for human use, yet *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast play a more practical role in farm animals (Simon et al., 2001). In recent decades, applications based on lactic acid bacteria (LAB) have increased, which is probably associated with development of better processing technologies (Jin et al., 2000; Gueimonde and Sánchez, 2012; Othman et al., 2017). Multiple criteria have been established and employed for the selection of proper candidate strains of probiotics (de Melo Pereira et al., 2018). The criteria mostly rely on their *in vitro* activity such as host-associated stress resistance, epithelial adhesion ability and antimicrobial activity (Araya et al., 2002). However, many probiotic products showed the additional advantage in improving animal growth performance in *in vivo* assessments (Frizzo et al., 2010; Vieira et al., 2013; Wang et al., 2017; Dowarah et al., 2018). The mode of action behind these effects are still unknown.

2.2.2 Phytobiotics

There is a long history of medicinal usage of herbal products for human and animals (Jamshidi-Kia et al., 2018). Bioactive compounds derived from plants that improve health and productivity of animal husbandry are referred to as phytogenic products or phytobiotics (Puvača et al., 2013). They originate from a variety of plants such as garlic, oregano, thyme, rosemary, coriander or cinnamon (Manafi et al., 2016) and can be prepared as whole plants or processed parts of plants including roots, leaves, barks or seeds (Hosna Hajati, 2014). Additionally, phytobiotics are usually formulated and used as individual phytochemical or blends of different ingredients (Gadde et al., 2017). However, it has been concluded that beneficial effects of phytobiotics may vary since it depends on the integrated effects of all constituents (Giannenas et al., 2013).

In general, substances can be categorized into two groups, primary and secondary substances. Primary substances are nutritional compounds such as protein or fat. Secondary ingredients are the bioactive extracts like volatile oils, bitters or other phytochemicals (Diaz-Sanchez et al., 2015). Compared to primary ingredients, the secondary substances are of more interests due to multiple nutritional functions. Many bioactive molecules such as carvacrol, thymol, cineole, linalool, anethole, allicin, capsaicin, allylisothiocynate, piperine and others have been identified as the functional substances with beneficial influence (Grashorn, 2010). Polyphenolic ingredients have been regarded as the main bioactive compounds, however, the composition and concentration of phenolic ingredients depend on plant type, parts of the plant used, geographical origin, harvesting season, environmental factors, storage conditions as well as processing techniques (Gadde et al., 2017). Therefore, a comparison between different

phytogenic products is difficult.

Phytobiotics have reportedly shown many desirable advantages not only for feed and animals but for animal-derived products as well (Windisch et al., 2008). Besides this, in-feed phytobiotics may also enhance palatability of diets (Randrianarivelo et al., 2010; Zeng et al., 2015). However, the palatability-refined character is not well applied to poultry nutrition since the birds are not sensitive to odors (Roper, 1999b). Similar to probiotics, many types of phytobiotics are also certificated as GRAS status with minor safety concerns (Prabakar et al., 2016; Thirumal et al., 2019). Considering all these characteristics, the phytobiotics are also ideal candidates as novel bioactive feed additive to improve animal production.

2.3 Growth promotion of animals by probiotics and phytobiotics as feed additives in poultry

2.3.1 Probiotics

The efficiency of various probiotic strains has been tested for different poultry species in different studies. Firstly, promotion of growth performance of healthy animals was most widely studied. Among all probiotics, *Bacillus* spp. and *Lactobacillus* spp. are most often used probiotic bacteria and many of them have demonstrated a positive impact on growth performance. As summarized in a review by Kabir, some probiotic strains are able to positively affect the performance in both active and inactive status from aspects of body weight gain and feed conversion ratio (Lutful, 2009). Specifically, several *Bacillus subtilis* strains were able to significantly improve animal performance by increasing the weight gain or decreasing the feed conversion ratio (FCR) in broiler chickens, laying hens, ducks and geese (Wu et al., 2008; Guo et al., 2016; Park et al., 2018; Neijat et al., 2019). Similarly, several *Lactobacillus* strains including *L. acidophilus*, *L. bulgaricus* or *L. salivarius* were found to enhance the performance of broilers when supplemented into diets (Apata, 2008; Forte et al., 2017; Shokryazdan et al., 2017). Moreover, non-bacterial microorganisms were also reported with probiotic properties that promote poultry production. In a newly published investigation, a probiotic yeast, *Diutina rugosa*, significantly increased the body weight (BW) during week 3 to week 6 and the feed conversion ratio (FCR) in early starter phase (Wang et al., 2019). Except being used as single-strain additive, a particular attention is drawn to the utilization of probiotic cocktails. For instance, dietary multi-strain probiotics containing *L. acidophilus*, *B. subtilis* and *C. butyticum* was reported to significantly result in the greater BW gain for broiler chickens than birds in control group especially in grower phase (Zhang and Kim, 2014). In this study, the researchers also found that probiotic cocktails enhanced ileal digestibility of most essential amino acids. The same beneficial effect was also observed when a combination of a bacterial probiotic cocktail (*L. acidophilus*, *B. subtilis*, and *A. oryzae*) and a non-bacterial probiotic (*S. cerevisiae*) was tested on broiler chickens. This probiotic complex increased bird BW and dressing

percentage, as well as better FCR compared to birds fed a basal feed (Hussein and Selim, 2018).

Some investigations studied the dynamic impact of probiotic additives on animals challenged with specific pathogens, revealing a scenario how feed additives may support birds under the condition of infection (challenge trial). In one study, a decreased average BW gain and an increased FCR were observed in the broilers challenged with *C. perfringens*. However, the supplementation of *L. acidophilus* in feed significantly increased the BW of the infected birds and reduced animal mortality (Li et al., 2018). The reduction of weight and increase in feed conversion triggered by pathogen infection has been one of the main concerns that cause economic loss in the poultry industry. Due to this fact, probiotics have been developed to compensate the weight reduction caused by pathogens like *E.coli* or *Salmonella* spp. (Abudabos et al., 2019; Ateya et al., 2019).

However, it has to be noticed that the administration of probiotic additives do not necessarily induce a better growth performance in poultry. Some studies also indicate that the used probiotic additives did not exert significant effects on improving growth performance (Lee et al., 2010; Zhang et al., 2011). Many factors such as host-specificity, in-feed concentration, preparation and processing of probiotics as well as rearing conditions tend to be overarching factors that influence the efficiency of probiotics.

2.3.2 Phytobiotics

The focus on replacing AGP with phytobiotics has intensified in the last decades and numerous investigations show that growth performance can be increased with phytobiotics. A number of reviews summarize these growth promoting effects in poultry (Yang et al., 2015; Mohammadi Gheisar and Kim, 2018). In these reviews, a number of different phytogenic feed additives improved average daily gain, feed conversion ratio and/or average daily feed intake of broilers or ducks. To broaden the understanding of the effects in details, some applications in different animals are given as below. In an experiment involving 900 broiler chickens with a natural blend herbal extracts (basil, caraway, laurel, lemon, oregano, sage, tea, and thyme), the supplementation of the phytobiotics significantly resulted in higher weight gain. Besides, the weight of carcass and breast percentage also improved significantly in the same study (Khattak et al., 2014). In another broiler trial, turmeric and garlic additives were added to a chicken diet. The results indicated that the single turmeric meal or combined with garlic meal significantly promoted body weight gain and feed conversion (Olukosi and Dono, 2014). Phytobiotics were also shown to compensate growth loss in pathogen-challenged animals. Xue and coworkers compared the growth performance in a challenge trial with *Clostridium perfringens* when plant-derived isoquinoline alkaloids (IQA) from *Macleaya cordata* were used (Xue et al., 2017). The IQA meal in that study significantly increased BW gain and feed intake on day 24 and 35,

moreover, the IQA also significantly improved FCR and breast yield. The studies on improvement on growth performance were as well reported in other poultry species. In a trial of meat-type ducks, researchers conducted a feed experiment on 160 ducklings for 42 d. The BW gain and FCR of ducks receiving a phytobiotic matrix (quillaja, anise, and thyme) in their diet showed a significantly higher level by 2.6% and 3.5% respectively from 21-42 d (Gheisar et al., 2015). Eggs have been long produced and utilized as one of best dietary protein source in animal husbandry (Kuang et al., 2018). Egg-producing poultry species have been reported with refined egg production responding to in-feed phytobiotics. In a study with laying hens, rosemary oil exerted a significant effect on laying rate and egg mass at a dose of 100 mg/kg feed (Garcia et al., 2019).

An attractive characteristic of phytobiotics which plays an important role in animal nutrition is their palatability (Kaschubek et al., 2018). The feed palatability demonstrates multiple benefits feed on their odor which might increase the feed intake of animals (Windisch et al., 2008). However, this promising characteristic has been observed in other farm animals like swine (Stelter et al., 2013), but only limited work for poultry is available. The rationale might be understood as poultry has generally a lack of sensitivity to odors (Roper, 1999a). As for feed intake, there are also studies reporting a significant decline in broilers and laying hens fed with dietary phytobiotics (Roth-Maier et al., 2005). Nevertheless, unlike higher possibility of feed refusal observed in mammals, poultry were reported to respond to low levels of spices in feed by increasing digestive secretions without significantly rejecting feed (i.e., decreasing feed intake)(Brenes and Roura, 2010).

In view of nutrient utilization and digestibility, phytobiotics reveal extensive advantages by elevating digestive capability. For instance, several essential oils were corroborated with development of the activities of trypsin, maltase and pancreatic amylase in broiler chicken (Lee et al., 2003; Jang et al., 2004; Jang et al., 2007).

2.4 Manipulation of gut microbiota by probiotic and phytobiotic additives in poultry

2.4.1 Probiotics

For in-feed probiotics, many publications suggest that health promoting effects are associated with modifications of the gut microbiota (Clavijo and Flórez, 2018; Rubio, 2018). Thus, the alteration of the animal microbiome has been always of interest since the gut microbiota strongly interacts with the host (Nicholson et al., 2012). In many studies, probiotic strains are reported to actively regulate the gut microbiota composition of several poultry species. For instance, two *Bacillus subtilis* strains were evaluated in broiler chicken and were found to significantly increase the relative abundance of *Pseudomonas*, *Burkholderia*, and *Prevotella* spp. in caecum of broiler chickens during the grower phase compared to the control group. Furthermore, the same two strains also demonstrated the ability to increase the diversity of the

jejunal microbiota (Li et al., 2019). In another investigation, *L. sakei* was supplemented to mule ducks during the overfeeding stage. The added *L. sakei* on one hand significantly decreased the relative abundance of *Bacteroides* in the ileum at the mid-overfeeding, on the other hand also decreased the ileal microbiota richness at from mid-overfeeding and cecal microbiota diversity at the end stage of overfeeding (Vasaï et al., 2014).

The further development of bioinformatic methods has deepened our understanding on the microbiome. Gao and colleagues monitored the patterns of microbiota development with a trained Random Forest model, which could identify age-discriminatory taxa. They found the supplementation of *L. plantarum* in feed was able to accelerate the maturation of the intestinal microbiota. On the contrary, the conventional in-feed AGP significantly decreased time-based intestinal microbiota development (Gao et al., 2017). The gender of birds has also been found to be a confounding factor in investigating the effects of in-feed probiotic to gut microbiota of animals, because the difference of microbiota composition between male and female birds were reported. Another study indicated that the microbiota composition similarity between males and females was observed under 30 % using denaturing gradient gel electrophoresis (DGGE) (Lumpkins et al., 2008). Since most microbiota studies only included the data collected from male birds or gender-unknown birds, the sex bias must be considered to deepen the understanding upon the interaction between microbiota shifts and probiotic inclusion (Kers et al., 2018a).

Additionally, several probiotics have the ability to inhibit a general or specific spectrum of microorganisms, often microorganisms with a negative impact on host health. For instance, the application of dietary *Enterococcus faecium* could reduce intestinal *Salmonella* in broiler chicken (Mohammadi Gheisar et al., 2016). Lavipan, a multi strain probiotic product, demonstrated a remarkable exclusion to *Campylobacter* spp. as feed additive of broiler chickens. This probiotic cocktail can not only decrease *Campylobacter* spp. infection in broilers, but can also reduce *Campylobacter* spp. contamination in chicken carcass (Smialek et al., 2018). Various modes of action were described to explain a probiotic-induced inhibition of bacterial pathogens. A classic mechanism is the principle of competitive exclusion (CE), which is mainly due to topographical competition for niches (for instance in the mucosal layers) as well as to competition for nutrients, by which pathogenic microorganisms are prevented from colonizing the intestinal tract (Patterson and Burkholder, 2003). CE was regarded as the most effective and harmless method available to control intestinal disturbances in poultry (Schneitz, 2005). Many studies implied the antagonistic effect based on CE to a wide range of bacteria including Extended-Spectrum-β-Lactamase (ESBL)-producing *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp. and others (Stern et al., 2001; Ceccarelli et al., 2017). Another noteworthy mechanism may be the production of antibacterial substances by probiotics. Those substances are generally categorized into three groups, namely organic

acids, bacteriocins and bacteriocin-like inhibitory substances. The production of these antagonists have been an important criteria for the probiotic selection and numerous investigations have shown their inhibitory activity in *in vitro* conditions (Bermudez-Brito et al., 2012; Saint-Cyr et al., 2016; Medina Fernández et al., 2019). However, a common scenario under practical conditions is that probiotic supplementation may not always be able to perform the inhibitory activity as they have shown in *in vitro* assays. The inconsistency can range from a high efficacy to no response (Chambers and Gong, 2011). Thus, this calls for a re-evaluation on their *in vivo* performance during the transition from benchtop research to practical application.

2.4.2 Phytobiotics

As to the gut microbiota of animals, the in-feed phytogenic compounds are always found to either decrease the intestinal bacterial counts or their corresponding activities of some specific bacteria, mainly contributed by their inhibitory activity on bacterial growth (Zhai et al., 2018). Several experiments evidenced this mode of action by showing reduced bacterial growth in a dose-dependent manner. When birds were grouped to receive increasing doses of phytobiotics in feed from 1gm/kg feed to 10 gm/kg feed, a dose dependent decrease of cecal total viable bacterial count was observed in chicken fed with phytobiotics (Ripon et al., 2019). Another study with ducks confirmed the inhibiting activity on intestinal bacteria, as the addition of an oregano based product significantly reduced coliforms, total anaerobes and lactase-negative *Enterobacteria* in the caecum (Abouelezz et al., 2019).

However, unlike a direct reduction of the total bacterial count in the gut, changes in the composition of the intestinal microbiota have been shown. The changes were dependent on the specific phytogenic application. Different phytogenic applications have been shown to differently modify the microbiota in poultry. Some phytobiotics are related to the modification of the two most dominant phyla, *Firmicutes* and *Bacteroidetes*, often significantly increasing the *Firmicutes/ Bacteroidetes* (F/B) ratio in caecal microbiota of animals (Lillehoj et al., 2018), and significantly increased beneficial bacteria like *Lactobacillus* spp. (Mohammadi Gheisar and Kim, 2018). Nevertheless, there are also investigations that report contrary results. For example, Zhu and coworkers observed a sharp decline in the genus *Lactobacillus* after phytobiotic (carvacrol/thymol mixture) inclusion into diets (Zhu et al., 2019a). This indicates that the effect of different phytogenic applications cannot be generalized since they may lead to different effects.

Regarding their promising antimicrobial activity, phytogenic feed additives are often used to reduce the colonization of certain pathogenic or undesirable bacteria which frequently and seriously affect the poultry industry. *C. perfringens*, the common cause of poultry necrotic enteritis (NE), was reported to be controlled in several cases when phytobiotics were included

in broiler feed (Mitsch et al., 2004; Diaz Carrasco et al., 2016). Phytobiotics like mint, thymol, and cinnamaldehyde were also determined to significantly reduce the amount of *Campylobacter* spp. in the caecum (Micciche et al., 2019). In-feed phytobiotics were also repeatedly investigated regarding their *in vivo* inhibition of other intestinal pathogens like *Salmonella* spp. or *E. coli* in poultry (Lillehoj et al., 2018). Most phytobiotics possess a certain degree of inhibitory selectivity as summarized in a review (Chambers and Gong, 2011). The existing studies suggest that Gram-negative bacteria seem to be generally more vulnerable than Gram-positive bacteria by phytobiotics (Lin et al., 2000; Si et al., 2006b).
However, like in probiotics, due to a lack of studies it is still unclear if these effects are directly induced by the presence of the phytobiotics or are an indirect effect of changes within the intestinal microbiota.

2.5 Immunoregulation by probiotics and phytobiotics in poultry

2.5.1 Probiotics

Numerous studies highlighted that dietary *Lactobacillus* spp. supplementation may exert beneficial effects on the host via modifying its immune system. The role of immune effector of in-feed probiotics has been recognized by the European food safety authority (ESFA) and has been summarized in a recent report commissioned by the EFSA (IRTA, 2015). This systematic report lists probiotic activities of several commonly used species including *Lactobacillus* spp., *Bacillus* spp., *Enterococcus* spp., *S. cerevisiae* and *Bifidobacterium* spp., which showed beneficial modification of goblet cell numbers, stained area, mean size and mucosal thickness as well as an increase in the expression of immune-regulatory genes and genes related to humoral immunity. However, different modes of actions were observed for probiotics in regulating the immune system in poultry. The probiotics were proven to modulate the host immune system directly or indirectly (Markowiak and Śliżewska, 2018). More than 98% all multicellular organisms hold an innate immune system (Kogut, 2009). In poultry, the hypothesis is that the innate immune system acts with higher efficiency than the adaptive immunity based on specific T cells and specific antibodies (Klasing, 2007). Several *Lactobacillus* strains were found to more effectively induce cytokines such as gamma interferon (IFN-γ), interleukin-12 (IL-12), IL-18 and transforming growth factor β4 (TGF-β4) in chickens (Brisbin et al., 2011). Some other probiotics were identified to up-regulate the immune-gene expression and the apoptosis in caecum of chicken, which led to a reduction of bacterial infections induced by intracellular pathogens (Higgins et al., 2011). The application of a probiotic *Lactobacillus reuteri and Lactobacillus johnsonii* strain as dietary supplementation up-regulated the preventive function of gut barrier, thereafter demonstrated by a higher immune response against *Salmonella enteritidis* infection in chicken (Van Coillie et al., 2007). Different than the innate immune system, the adaptive immune system is specifically acquired when animals

encounter unknown antigens. The inclusion of probiotics to animals was shown to aid the adaptive immune system by increasing the antibody response or phagocytic activity (Yirga, 2015). The immune system of broiler chickens responded to a *L. plantarum* strain by higher concentrations of serum IgG and intestinal secretory IgA (Gao et al., 2017). Regarding the responses to specific antigens, there is also strong evidence that probiotics significantly increase the antibody level against a broad spectrum of gut pathogens including Newcastle disease virus (NDV), bronchitis, avian influenza virus (AIV), *Salmonella Typhimurium* and other antigens (Rowghani et al., 2007; Szabó et al., 2009; Sikandar et al., 2017). Despite the functional improvement described above, probiotics have been shown to regulate the immune system indirectly by increasing the weight of immune organs, regulating cytokine profiles and modifying toll-like receptors (TLRs) (Villena et al., 2008; Alkhalf et al., 2010; Asgari et al., 2018).

2.5.2 Phytobiotics

An increasing number of studies endorse the positive role of phytobiotics in modulating the poultry immune system. The addition of phytobiotics is reported to enhance the immune response of both the innate- and the adaptive immune system similar to probiotics. For instance, a phytobiotic derived from the turmeric plant was supplemented to broiler chickens for seven weeks in powder form, displaying a general elevation of antibody levels including IgA, IgG, and IgM as well as decreased ratio of monocytes in total blood cells (Emadi and Kermanshahi, 2007). Pathogen-specific antibody titers increased by phytogenic additives is reported for poultry species (broiler chickens, laying hens and etc.) against NDV, AIV, *Eimeria* microneme protein and infectious bursal disease virus (IBDV) (Böhmer, 2009; Landy et al., 2011; Özek et al., 2011; Kim et al., 2013). Another thyme essential oil provoked higher levels of lymphocytes and white blood cells after 42 d feeding in broilers (Hassan and Awad, 2017). On the other hand, several publications demonstrate a direct or indirect reinforcement of innate immunity by phytobiotics. A previous review has comprehensively summarized the functional members of phytobiotics and their possible modulatory actions on innate immunity (Huang and Lee, 2018). In that review, applications based on carvacrol, cinnamaldehyde, curcumin and thymol are described as being able to regulate the chicken innate immune system majorly via TLRs, mitogen-activated protein kinases (MAPKs) and the nuclear factor kappa B (NFκB) pathway. Another important pathway of phytobiotic-based immunostimulation is the modification of GIT cytokine expression. The supplementation of phytogenic cinnamaldehyde to newly hatched chicks for instance led to a remarkable rise on gene transcripts encoding interleukin (IL)-1β, IL-6, IL-15 and interferon (IFN)-γ in intestinal lymphocytes. The up-regulation was about 47 times higher compared to chicks received a basal diet (Kim et al., 2010).

Unlike probiotics, phytobiotics have commonly been used as extracts from plants, but also sometimes as sole active ingredients. The immune-modulatory effects of phytobiotics and their single compounds were found to be inconsistent in some studies (Lin et al., 2006; Lin et al., 2011). However, functioning substances have been mostly attributed to the secondary products of phytobiotics (Hashemi and Davoodi, 2010).

Whereas, continuous feeding of an immunomodulatory additive throughout the growing phase of poultry does not necessarily result in the over-stimulation of birds and therefore suffering from performance reduction (Swaggerty et al., 2019). Improved growth performance may go along with the immune system modification induced by inclusion of phytobiotics, which allows the reallocation of energy toward improving performance (Murugesan et al., 2015).

2.6 The production and formulation of pro-/phytobiotic as feed additives in poultry

2.6.1 Probiotics

Probiotics act in the host as living microorganisms. Thus, a sufficient viable cell number probiotics is a prerequisite condition for their function (Simon, 2005). To ensure viability and stability of probiotics in feed additives, its biomass must be protected after manufacturing. The most commonly utilized preparation is the drying method, which facilitates probiotics storage, handling as well as transportation in a stable form (Santivarangkna et al., 2008). The most popular techniques are freeze-drying and spray-drying to dehydrate the fermented probiotic culture, although there are also other methods like vacuum drying or fluid bed drying (Betoret et al., 2003; Broeckx et al., 2016). These two popular methods have their own merits and limitations, respectively. Compared to freeze drying, spray drying is more cost-effective, but always results in lower viability of the processed probiotics due to the exposure to high temperatures (Menshutina et al., 2010). Although there are several studies reporting the use of spray drying for in-feed formulation (Corcoran et al., 2004; Shokri et al., 2015; Zhu et al., 2016), freeze drying preparation is preferred for probiotics like *Lactobacillus* or *Bifidobacterium* spp. due to their lower resilience against heat processing (Chávez and Ledeboer, 2007). However, spore forming probiotics like bacilli are deemed more stable during processing and storage (Elshaghabee et al., 2017; Liu et al., 2018). The thermostability of their spore is fundamentally contributed by dipicolinic acid (DPA) (Setlow, 2006). On the contrary, freeze drying yields more viable cells by avoiding heat stress at a lethal level. However, considering its cost, freeze drying is generally regarded as an expensive method for maintenance. Furthermore, other factors such as material use, volume capacity and time of duration also increase costs compared to other methods (Peighambardoust et al., 2011).

No matter which method is applied for the formulation of probiotics, the potential viability loss cannot be neglected, especially on an industrial scale. Therefore, necessary optimizations of processes have to be conducted to minimize loss of viability (King and Su, 1993; Fu and Chen,

2011). The mainstream strategy is to develop a protective- or carrier system against external stress factors. Numerous protectants to stabilize the probiotic during freeze/ spray drying have been studied. The supplementation of sugars, especially of the disaccharide type, into probiotic formulations have shown a promotion of viability of many probiotic strains after the drying process (Santivarangkna et al., 2008; Broeckx et al., 2016). As examples, sugars like trehalose, sucrose, maltose or lactose were extensively used as protectants for probiotic formulations (Miao et al., 2008; Behboudi-Jobbehdar et al., 2013). Protein-based stabilizers are another important source of drying protectants. The effect of different protein protectants (e.g. skim milk, sodium caseinate, whey) on cell viability of a *Lactobacillus acidophilus* strain during the spray drying process showed that whey protein exhibited maximum protection (Soukoulis et al., 2014). Similar protective effects of protein-based protectants were also observed for freeze drying of probiotics (Wong et al., 2010). To achieve a maximum protection during probiotic processing, the combined protectants matrix has become a prior option for its synergistic effects. With the advancement of analytical methods, the utilization of the response surface method (RSM), an alternative to the traditional one-variable-at-a-time approach strategy. With the RSM, potential interactions among different treatments are considered simultaneously (Huang et al., 2006). A determination of optimized protective media with RSM has been reported to stabilize probiotic lactobacilli in processing and storage (Ren et al., 2019). Next to protective media, the encapsulation of probiotics is another enhanced preservative means. Generally, encapsulation of probiotics immobilizes probiotic strains within semipermeable and biocompatible materials and protects against loss of viability (Gbassi and Vandamme, 2012). Additionally, encapsulation may also improve survival rate of the probiotic product during gastric transition and a better colonization can thus be obviously expected (Shori, 2017).

2.6.2 Phytobiotics

Phytobiotics are plant-derived products and thus the preparation of phytobiotics involves extraction and formulation of bioactive compounds out of the plants or microalgae (Grashorn, 2010). Phytobiotics are generally extracted from raw materials by infusing phytochemicals of interests in relevant solvents (Applegate et al., 2010). Phytobiotics can be formulated into solid form or used as crude/ concentrated extracts in liquid form depending on the bioactive ingredients (Gadde et al., 2017). Although there are applications of phytobiotic feed additives produced by vacuum drying or spray drying (Nm et al., 2018), freeze drying is still assumed to be more preferred for the preparation (Abascal et al., 2005). However, dried phytogenic extracts are still sometimes poorly conserved, as is the case for volatile, phenolic or carotenoid compounds (Abascal et al., 2005). The formulation in liquid phase, normally as an emulsion of oil or resin, are preferred for preserving the volatile or polyphenolic compounds.

Compared to probiotics, phytobiotic additives are more stable substances during processing. However, there are still several chemical factors impacting the stability of phytobiotics (Turek and Stintzing, 2013). These factors including light (both UV- and visible light), temperature, oxygen availability, metal contaminants, water content and compound structure, summarized and discussed comprehensively in a review (Turek and Stintzing, 2013). These factors may physically deactivate bioactive ingredients or induce reactions such as oxidation and polymerization, subsequently resulting in a loss of quality and pharmacological properties of phytobiotics. Therefore, some studies addressed these problems to develop protective formulations to steady the functionality of phytobiotic products. One study on phytobiotics for piglets suggests that carvacrol and thymol phytobiotic coated with celite or alphacel led to better stability (Michiels et al., 2010). Encapsulation technologies are frequently used for phytobiotics. Phytobiotics may absorb to feed components or in the upper small intestine of animals, and thus decrease established doses or weaken their corresponding bioactivity in the lower gut (Kohlert et al., 2000; Si et al., 2006a; Michiels et al., 2008). Hence, encapsulation plays an important role in the protection as well as in the controlled release at targeted locations in the intestine.

2.7 Synergistic combinations of probiotic and phytobiotic additives in poultry

It is not rare that different types of feed additives have been used as combinations to invoke synergistic effects, which may lead to greater response than their single application (Shipradeep et al., 2012). This principle has been mostly applied to the combination of probiotics and prebiotics, namely the synbiotics. The beneficial effect of synbiotics has been implied as the promotion of beneficial bacterial fermentation with supplementation of suitable substrates (de Vrese and Schrezenmeir, 2008). Synbiotics enjoy the merits from both probiotics and prebiotics, and are developed to overcome some possible difficulties in the survival of probiotics in the gastrointestinal tract (Rioux et al., 2005). Thus, most additive combinations were designed following this synbiotic concept. Different combinations with different probiotics and prebiotics were studied under research conditions and in practical case studies. Several investigations evidenced the effects of synbiotics on improving body weight, carcass quality, immune system as well as the net profit of poultry production. Apart from prebiotics, combinations with other feed additives such as acidifiers or enzymes were also reported (Seifi, 2014; Rodjan et al., 2018).

Phytobiotics are sometimes combined with other feed additives to enhance or compensate their activity as well. In some studies, phytobiotics in combination with organic acids were found with greater benefits on improving BW gain, FCR or antibacterial profile in poultry (Mikulski et al., 2008; Bozkurt et al., 2012; Gilani et al., 2018). The synergistic effects of phytobiotics and acidifiers are not well understood yet. However, several studies revealed some potential

modes of actions, for instance that phenolic substances in phytobiotics may induce permeabilization of bacterial cell membranes and thus increase the susceptibility of bacteria to organic acids. On the other hand, a decreased pH by acidifiers may result in higher phytobiotic hydrophobicity, which subsequently increases the possibility of phytobiotics to penetrate through the lipid content of bacterial membranes (Karatzas et al., 2001). In one study, combinations of enzymes with phytobiotics similarly improved the performance more than the single use of phytobiotics in chicken nutrition (Diaz-Sanchez et al., 2015).

As noted, phytobiotics tend to selectively inhibit the bacterial growth or activity (Zhai et al., 2018), while probiotics tend to modulate the intestinal microbiota without selective inhibition. Thus, expecting synergism for probiotic and phytobiotic combinations seems contradictory at first glance. However, due to the complex nature of the host-microbiota interactions, synergistic effects may not be as straight forward as just adding up, in this case, the effects of single feed additives. Synergistic effects may also occur for members of the gut microbiota that were not modified by supplementation of individual components. There are only a quite limited number of examples for the combination of phytobiotics with probiotics. A commercial phytobiotic and a probiotic *E. faecium* strain were blended as mix additive and tested on broiler chickens. The results indicated that the probiotic-phytobiotic combination promoted animal health but did not significantly improve animal performance (Erdoğan et al., 2010). Another example is that phytobiotics and probiotics can be formulated into a complex additive matrix with other feed additives for synergistic effect based on multiple ingredients. One investigation prepared a matrix supplementation consisting of a probiotic, a prebiotic and a phytobiotic. This formulation significantly improved the FCR of chickens suffering from *C. perfringens* induced necrotic enteritis during the starter and grower phase (Calik et al., 2019). A recently published investigation compared the combination of a probiotic and a phytobiotic to their single application as well as to antibiotic supplementation. In that study, the body weight gain in the combination group was lower than in the single probiotic group but higher than in all other groups. In the light of microbial and hematological performance, the combination was not superior to other experimental- or control groups (Ferdous et al., 2019).

There are also few cases providing the practical effect of probiotic-phytobiotic combinations as feed additives to other livestock (Nowak et al., 2017). However, there is still a lack of understanding for probiotic-phytobiotic combinations and their potential synergic effect in poultry nutrition. The commonly used probiotic bacteria, for example *Lactobacillus* spp., *Bacillus subtilis* or *Bifidobacterium* spp., belong to Gram-positive bacteria, which are usually tolerant to phytogenic substances *in vitro* (Lin et al., 2000). The question of how probiotics, especially host-specific probiotics, react to co-supplemention with phytobiotics in their respective host is yet unclear since both increase and decrease of probiotics have both been reported in feeding trials (Mohammadi Gheisar and Kim, 2018; Zhu et al., 2019b). Thus, more

in-depth studies regarding the effect of probiotic-phytobiotic combinations are necessary to convey a better knowledge on their alternative role in the replacement of AGP.

CHAPTER 3: AIMS AND OBJECTIVES

The aim this thesis was to isolate and produce host-specific probiotics and to investigate the possible synergistic effects of these probiotics with commercial phytobiotics. The thesis includes the following objectives:

1. Establishment of a rapid screening method for host-specific *Lactobacillus* probiotics;
2. *In vitro* characterization of the selected candidate strains;
3. Determination of growth conditions of the candidate strains;
4. Optimization of cryo-protectants and evaluation of storage stability;
5. Proof-of-principle feeding trial with candidate strains and phytobiotics with in-depth analysis of the gut microbiota;

The results of current thesis have been included and reported in the published manuscripts in the following chapters (**Chapter 4**, **Chapter 5** and **Chapter 6**).

CHAPTER 4: Screening of Host Specific Lactic Acid Bacteria Active Against *Escherichia coli* From Massive Sample Pools With a Combination of *In Vitro* and *Ex Vivo* Methods

H. Ren*, E-M Saliu, J. Zentek, F. Goodarzi Boroojeni, W. Vahjen

Institute of Animal Nutrition, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany.
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*Correspondence: hao.ren@fu-berlin.de; Tel.: +49 30 83850149

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Screening of Host Specific Lactic Acid Bacteria Active Against *Escherichia coli* From Massive Sample Pools With a Combination of *in vitro* and *ex vivo* Methods

Hao Ren*, Eva-Maria Saliu, Jürgen Zentek, Farshad Goodarzi Boroojeni and Wilfried Vahjen

Institute of Animal Nutrition, Freie Universität Berlin, Berlin, Germany

A novel three-step combination of *in vitro* and *ex vivo* screening was established to massively screen host derived lactic acid bacteria (LAB) from the broiler chicken intestine with inhibitory activity against *Escherichia coli*. In a first step, a massive sample pool consisting of 7102 broiler-derived colonies from intestinal contents were established and sub-cultured. Supernatants thereof were incubated with an *E. coli* model strain to screen suitable isolates with inhibitory activity. A total of 76 isolates of interest were subsequently further studied based on either pH dependent or -independent activity in the second step of the assay. Here, in-depth growth inhibition of the *E. coli* model strain and the potential of isolates for lactic acid production as inhibitory substance were indexed for all isolates. Resulting scatter plots of both parameters revealed five isolates with exceptional inhibitory activity that were further studied under *ex vivo* condition in the third step of the assay. These isolates were taxonomically classified as strains of the species *Lactobacillus agilis*, *Lactobacillus salivarius*, and *Pediococcus acidilactici*. Samples from the broiler chicken intestine were inoculated with the *Lactobacillus* isolates and the *E. coli* model strain. After 8 and 24 h incubation, respectively, growth of the *E. coli* model strain was monitored by cultivation of the *E. coli* strain in antibiotic supplemented medium. By their superior inhibitory activity against the *E. coli* model strain, one *L. agilis* and one *L. salivarius* strain were selected and characterized for further application as probiotics in broiler chicken. Additionally, their antibiotic resistance patterns and resilience under gastric stress of isolates were also characterized. The results of this study demonstrate that the novel isolation procedure was able to efficiently and rapidly isolate and identify bacterial strains from a massive sample pool with inhibitory potential against specific types of bacteria (here *E. coli*). The introduction of the final *ex vivo* selection step additionally confirmed the inhibitory activity of the strains under conditions simulating the intestinal tract of the host. Furthermore, this method revealed a general potential for the isolation of antagonistic strains that active against other pathogenic bacteria with specific biomarker.

Keywords: probiotics, lactic acid bacteria, host-derived, effective screening, *E. coli*, *ex vivo* model, massive sample pool

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Vittorio Capozzi,
University of Foggia, Italy

Reviewed by:
Elena Bartkiene,
Lithuanian University of Health Sciences, Lithuania
Prakash M. Halami,
Central Food Technological Research Institute (CSIR), India

****Correspondence:***
Hao Ren
hao.ren@fu-berlin.de

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INTRODUCTION

The search for alternatives to antibiotics is an important topic worldwide. Various groups of feed additives have been studied so far and probiotics seem to be promising candidates to increase animal health and performance in the absence of in-feed growth promoters (Mehdi et al., 2018).

As defined by Food and Agriculture Organization/World Health Organization (FAO/WHO), probiotics are "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Fao-Who., 2006). However, in the field of animal nutrition, especially for farm animals, probiotics are to protect the animal against specific pathogenic bacteria or have beneficial effects on animal performance (Chaucheyras-Durand and Durand, 2010; Liao and Nyachoti, 2017; Markowiak and Śliżewska, 2018).

As a group of extensively studied probiotic, lactic acid bacteria (LAB) have demonstrated inhibitory effects on certain microorganisms and potentially benefits on animal health (Dowarah et al., 2017). A large body of evidence have shown that LAB strains can exert beneficial impact by regulating intestinal inflammation or decreasing colonization of zoonotic bacteria like *Escherichia coli*, *Campylobacter jejuni* or *Salmonella enterica* (Santini et al., 2010; Vasanth et al., 2015; Azizkhani and Tooryan, 2016; Forkus et al., 2017; Wang et al., 2017). Among investigated pathogens, *E. coli* is one of the most well-documented target, and numerous investigations show eÿciency of LAB on inhibiting *E. coli* growth or preventing *E. coli* infection (Sherman et al., 2005; Kimble et al., 2015; Azizkhani and Tooryan, 2016). Therefore, LAB have been also intensively studied and widely used in recent decades for their beneficial properties as potential antagonists (Kajander et al., 2005; Hong et al., 2014; Lan et al., 2016). Diverse LAB products have been developed on the basis of wide array of species including *L. reuteri*, *L. acidophilus*, *L. intestinalis*, *L. plantarum*, *L. casei*, and *L. sakei* (Kılıç and Karahan, 2010; Karami et al., 2017; Tashakor et al., 2017). The actual isolation of probiotic bacteria is a field of research that has not been addressed in depth so far. Theoretical selection criteria for probiotics including LAB for human use recommended by the WHO include host-related stress resistance, epithelial adhesion and antibacterial activity as well as biosafety (Zhang et al., 2016; de Melo Pereira et al., 2018). Other parameters such as aggregative ability, hydrophobic phenotyping, reduction of pathogenic virulence, immunomodulation and specific metabolic pathway were also reported as possible criteria for selection (Saint-Cyr et al., 2016). *In vitro* criteria are preferred because of simplicity and cost-eÿciency (Papadimitriou et al., 2015). However, the characterization of probiotic LAB strains by using *in vitro* methods alone may not be suÿcient to predict their *in vivo* scenario, as different bacterial strains may behave differently under the conditions of the intestinal tract (Murima et al., 2014). Whether the selected LAB are able to colonize the host is as well an essential question. On the other hand, *in vivo* selection procedures are time-consuming, costly and carry ethical considerations, even though it offers the most direct impact of probiotic on host animals at given condition (Martins et al., 2008). This implies that an eÿcient screening assay for potential probiotic bacteria should include the steps to mimic *in vivo* conditions and at the same time be feasible in terms of laboratory work.

Commonly, the number of isolates screened for probiotic activity were comparably low, ranging between 14 and 1150 isolates with the majority of studies using only 50 to 80 isolates (Robyn et al., 2012; Babot et al., 2014). Thus, to our best knowledge, there is no published method to massively screen bacterial isolates with specific antibacterial activity. Considering the vast diversity of bacterial species in the intestinal tract as well as the occurrence of numerous strains in each species, it seems promising to screen as many potential probiotic isolates as possible to increase the probability of success. Also, the origin of probiotic has not yet been considered as significant factor previously. However, the advantage of isolation of host-specific probiotics become increasingly focused because those strains have already shown the capability to colonize the hosts (Zmora et al., 2018).

Most existing studies on probiotic LAB focus solely on their antagonistic activity in *in vitro* (Gram and Ringø, 2005). Recently, it was hypothesized that the intestinal tract of poultry harbors strains capable to inhibit the inhabitation of potential pathogens (Nhung et al., 2017; Shang et al., 2018). Therefore, the present study developed a three-step combination of *in vitro* and *ex vivo* methods to massively screen LAB isolates for their potential to inhibit *E. coli*. The final *ex vivo* model confirmed inhibiting activity under conditions simulating the gastro intestinal tract simultaneously as it is more easily controlled. Due to the technical simplicity of this method, it has the general potential for the development of other probiotics that target specific bacteria.

MATERIALS AND METHODS

Strains and Media

Throughout the study, an extended-spectrum beta-lactamase producing *E. coli* strain ESBL10716 (phylotype B1) was used as a model strain. It was isolated from excreta samples of broiler chicken by the Institute of Microbiology and Epizootics of Freie Universität Berlin within the RESET program and produces the CTX-M-15 lactamase (Falgenhauer et al., 2016). The resistance of model strain against cefotaxime was used as a specific marker in all culture and growth experiments. The strain was selected as a representative target strain from 13 *E. coli* strains of broiler origin in a pre-experiment, showing the strongest resistance against *in vitro* GIT stress and stress of random LAB supernatants (data not shown). The strain was stored as cryo stock and cultured in brain heart infusion broth (BHI, Carl Roth GmbH + Co., KG, Germany) for further application.

Sampling and Original Isolation

Intestinal samples were taken from broiler chicken (Cobb500). Fresh digesta samples from the crop, ileum, jejunum and cecum and excreta were obtained from different feeding trials conducted at the Institute of Animal Nutrition, Freie Universität Berlin and immediately processed. The animals received standard basal feed

with no zootechnical feed additives. Samples were serially diluted in Phosphate Buffered Saline (PBS, Sigma-Aldrich, Chemie GmbH, Germany) buffer, pH 7.4 and plated on de Man, Rogosa, and Sharpe (MRS, Carl Roth GmbH + Co., KG, Germany) agar plates. After anaerobic growth at 39°C for 48 h, single colonies from different dilutions and with different colony morphologies were picked with sterile toothpicks into microtiter plates supplemented with MRS broth (Carl Roth GmbH + Co., KG, Germany). Supernatants of colonies with visible growth were subcultured in microtiter plates. The original plates were kept at 4°C until after the preliminary screening (max. 48 h). Isolates of interest after the first screening were preserved from microtiter plates to cryo stock in −80°C freezer.

Ethical Statement

Samples were taken from studies that were conducted in accordance with the German Animal Welfare Act (TierSchG) and approved by the local state oÿce of occupational health and technical safety "Landesamt für Gesundheit und Soziales, Berlin" (LaGeSo Reg. Nr. T 0162/16 and A 0100/13).

Step 1: Massive Isolation and Preliminary Screening of Intestinal Lactobacilli

In the first step, a large samplepool was established and subjected to a pre-screening system.

Buffering and Deacidification Filtering (Pre-screening)

Regarding the initial pre-screening, two different approaches were tested to rapidly screen a large number of isolate supernatants. Thus, before inoculation of the *E. coli* model strain, one subset of supernatants (2208 isolates) was mixed with same volume of double strength BHI medium buffered with 0.4 M citrate buffer (pH = 6.2, Sigma-Aldrich, Chemie GmbH, Germany), while another subset of supernatants (2592 isolates) was supplemented with 3.5 µL 5 M NaOH (Carl Roth GmbH + Co., KG, Germany). Optimal buffering and deacidification conditions that still allowed growth of the *E. coli* model strain were determined in a series of pre-experiments (results shown in **Supplementary Materials**). The microtiter plates were inoculated with 10 µL *E. coli* culture (10^4 CFU/mL and incubated overnight aerobically at 37°C. Final optical density (OD) was read with a microtiter plate reader at 690 nm (Tecan Infinite200Pro, Germany) to determine bacterial growth. The final OD was used as indicator of inhibitory potential of a given isolate.

Step 2: *In vitro* Selection

The second step of the screening studied the *E. coli* growth inhibition in depth via growth curves in combination with lactic acid production of the isolates as probable inhibitory substance.

For this purpose, supernatants were generated by inoculating the LAB candidates at 10^4 CFU/mL in 10 mL MRS medium and incubated anaerobically at 39°C for 48 h. Supernatants were either used as is or adjusted to pH 6.5 with 5 M NaOH.

Lag time for *E. coli* growth was chosen as the first inhibition-related parameter and assessed according to previous study with necessary modification. In brief, pH-neutralized supernatants of the isolates were combined with same volume of double strength BHI medium and then dispensed into microtiter plates at 190 µL per well. The model *E. coli* strain (10 µL) was added to each well yielding a final concentration of 10^4 *E. coli* cells/mL. Cultures were then incubated aerobically at 37°C and turbidity (OD_{690nm}) was recorded every 5 min for 24 h using a microtiter plate reader (Tecan Infinite200Pro, Germany). Resulting growth curves were analyzed for lag time against respective controls without supernatants using the 3-parameter sigmoidal equation for bacterial growth and compared to respective controls. All growth experiments were carried out in triplicate. Lactic acid production was measured as aother probable inhibitory parameter. Triplicates of non-pH controlled supernatants were prepared as described above. Protein was precipitated by Carrez solution, the supernatant was filtered (0.45 µm filter, Carl Roth GmbH + Co., KG, Germany) and the concentration of lactic acid was measured with an enzymatic test reagents (R-Biopharm AG, Germany) according to the manual with minor modification. The L-/D-lactic acid standards were prepared with diluting pure L-/D-lactic acid to a serial dilutions (0, 26.5, 53, 79.5, 132.5, 185.5, 238.5, and 265 mg/L) and treated supernatant of each isolate was 1:50 diluted. 10 µL of each sample was added to 200 µL reagent 1 (L-/D-lactic acid-dehydrogenase buffer) and incubated at room temperature for 3 min. 10 µL distilled water was also incubated as reagent blank (RB). The OD was read once as A_1 after the incubation, then 50 µL reagent 2 (NAD solution) was added to each reaction. The samples were again incubated in room temperature for 15 min, then the absorbance was measured again as A_2. The standard curve was established with adjusted OD absorbance of all standards with equation "$\Delta A = (A_2\text{-}0.808A_1)_{Sample}\text{-}(A_2\text{-}0.808A_1)_{RB.}$" The standard curve for both L-lactic acid and D-lactic acid were plotted accordingly (calibration curves are shown in the **Supplementary Figures 4**, **5**). The concentration of each sample was further calculated with their corresponding adjusted OD by the standard curve.

To make the data comparable, the results of lag time and concentrations of lactic acids were indexed as follows: each read of lag time and lactic acid concentration was divided by the maximum value of the data set (lag time $_n$/lag time $_{max}$ or lactic acid $_n$/lactic acid $_{max}$) to reflect individual lag time extension/lactic acid production level among all tested isolates. Supernatants with superior lag time- and lactic acid index were then introduced to the final step of the isolation assay.

Step 3: *Ex vivo* Selection

An *ex vivo* model was prepared on the basis of a published method with minor modification (Starke et al., 2013) to test the impact of the chosen isolates on the survival of the *E. coli* model strain under conditions that are similar to the intestinal tract. Briefly, fresh digesta samples from the crop, jejunum or ileum were diluted 1:2 (w/v) with sterilized water. After sedimentation for 5 min, the supernatant of this suspension was transferred to sterile 15 mL tubes and dispensed into microtiter plates. LAB candidates (final concentration 10^7 CFU/mL) and the *E. coli* model strain (final concentration 10^4 CFU/mL) were then inoculated in triplicate. Non-inoculated suspensions served

as controls. All suspensions were incubated anaerobically at 37°C. This lower temperature than under *in vivo* conditions was chosen to chosen to allow the *E. coli* strain a better survival and therefore better detectability, as results show that even at 37°C the most active isolates completely inhibited *E. coli* survival after 24 h. Samples (10 μL) were obtained after 8 and 24 h incubation, respectively, and inoculated into cefotaxime (8 μg/mL, Thermo Fisher GmbH, Germany) containing BHI agar plates. After growth, colony forming units (CFU) as well as growth curves were analyzed as described above.

The three consecutive steps of screening are schematically shown in **Figure 1**.

Eligibility Criteria in Each Step

Isolates that showed inhibitory activity against the *E. coli* strain were determined after each step of the procedure. In the first step (massive screening), the end-point OD of *E. coli* culture co-incubated with pre-treated supernatants (buffered/deacidified) of LAB isolates were referred as the indicator to estimate growth inhibition of the *E. coli* strain. A cut-off value of 0.2 at OD_{690nm}, corrected for controls, was set as the threshold for growth inhibition. A total of 76 isolates were eligible for the next step.

In the second step, both lag time and lactic acid production were indexed. A scatter plot of these indices revealed those isolates with superior inhibition/concentration. The best five isolates were selected for the final step.

In the third step, isolates that induced the lowest *E. coli* survival in both growth assay and CFU after incubation under *ex vivo* conditions were selected as the final candidates.

Characterization of Selected Candidates

Taxonomic Identification of Candidates

Selected LAB isolates showing successful inhibition were identified on the species level via full length 16S rDNA sequence analysis using the classic universal primer pair F27 and R1492 by DSMZ(German Collection of Microorganisms and Cell Cultures, Germany) according previously published method (Stackebrandt et al., 2002).

Production of SCFA

Short-chain fatty acid in the supernatants of the candidates was analyzed via gas chromatography (Agilent Technologies 6890 N coupled with auto sampler G2614A and auto injector G2613A; Santa Clara, CA, United States). A total of 500 μl of each sample was mixed with the same volume of a CuSO4 solution (0.5 mmol/L). Protein in samples were precipitated by addition of 200 μl Carrez solution and centrifugation. After centrifugation, the samples were filtered through a 0.45 μm cellulose acetate (CA) filter and diluted with 0.5 mmol/L CuSO4 by 1:40 (v/v) for measuring. SCFA were then determined with a previously published method with minor modification (Schäfer, 1995). An Agilent 19095N-123 HP-INNOWAX polyethylene glycol column was employed in this experiment.

Aggregation Assessment

The auto-/co-aggregation abilities of selected LAB isolates were evaluated by a reported method with minor modification (Collado et al., 2008). Briefly, for auto-aggregation, stationary phase cultures were centrifuged (3 min, 10000 *g*, 4°C) and washed three times in PBS. The centrifugates were then re-suspended in PBS to an OD_{690nm} of 0.25 ± 0.05 (comparable to 10^7 – 10^8 cells/mL). Turbidity was measured every 2 h. To determine the auto-aggregation of strains, turbidity was calculated by the following equation: Auto-aggregation (%) = $1-(OD_t-OD_0) \times 100$ (where OD_t was absorbance after 2 h; OD_0 was the initial absorbance). For the co-aggregation, the centrifugates of lactobacilli isolates and tested *E. coli* were both processed and prepared as described above. Lactobacilli isolates and model *E. coli* were mixed at equal volume (vol/vol). Turbidity was monitored as described above and the co-aggregation rate was calculated by: $[(OD_E + OD_L)/2-(OD_{CO})/(OD_{ESBL} + OD_{LAB})/2] \times 100$ (where OD_E was the *E. coli* control; OD_L was the lactobacilli isolate control and OD_{CO} expresses the turbidity of coincubation).

Tolerance of Isolates to Acid, Osmotic Pressure and Bile

The tolerance of lactobacilli isolates to acid and bile stress was assessed by their viability and growth under conditions encountered in the stomach and small intestine, respectively.

Overnight cultures were centrifuged (3 min, 10000 *g*, 4°C) and washed three times with PBS, pH 7.0. The centrifugates were diluted to 10^8 cells/mL then inoculated into acidified MRS broth at pH 2, 3, and 4 or MRS broth supplemented with bile salt (w/v: 0.1%/0.3%/0.5%/0.7%) in a microtiter plate, respectively, and incubated anaerobically overnight at 39°C. Turbidity (OD_{690nm}) was monitored every 5 min and growth curves were plotted accordingly. Another set of centrifuges of the same cultures was diluted with pH-adjusted incubation buffer and bile containing buffer to approximately $\log_{10}$ 8.0 cells/mL and incubated at 39°C for 6 h. Samples from incubations were taken every 2 h and viable cells were enumerated by plating.

Tolerance against osmotic pressure was assessed with a published protocol with minor modification (Ng et al., 2015). After overnight incubation (anaerobically, 39°C), cultures were centrifugates (3 min, 10000 *g*, 4°C), washed in PBS buffer and 10^9 cells/mL were inoculated in MRS broth supplemented with sodium chloride of 2–10% final concentration. End-point turbidity at OD_{690nm} was determined after 40 h and compared to respective controls.

Adhesion and Competitive Adhesion Assay

The *in vitro* adhesion assay was performed according to previous report with minor modifications (Yeo et al., 2016). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12, Merck, Germany) supplied with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and amphotericin B (0.5 μg/mL) under 5% CO_2 in a 95% air atmosphere with 90% humidity at 37°C. The cells were then seeded onto 12 well plates (Greiner Bio-one GmbH, Germany) with of 2×10^5 cell per well in antibiotic free medium. After confluence of cells reached approximately 80%, the cells were exposed to 10^8 CFU lactobacilli candidate or combination of 10^8 CFU lactobacilli candidates with 10^7 CFU model *E. coli*. After

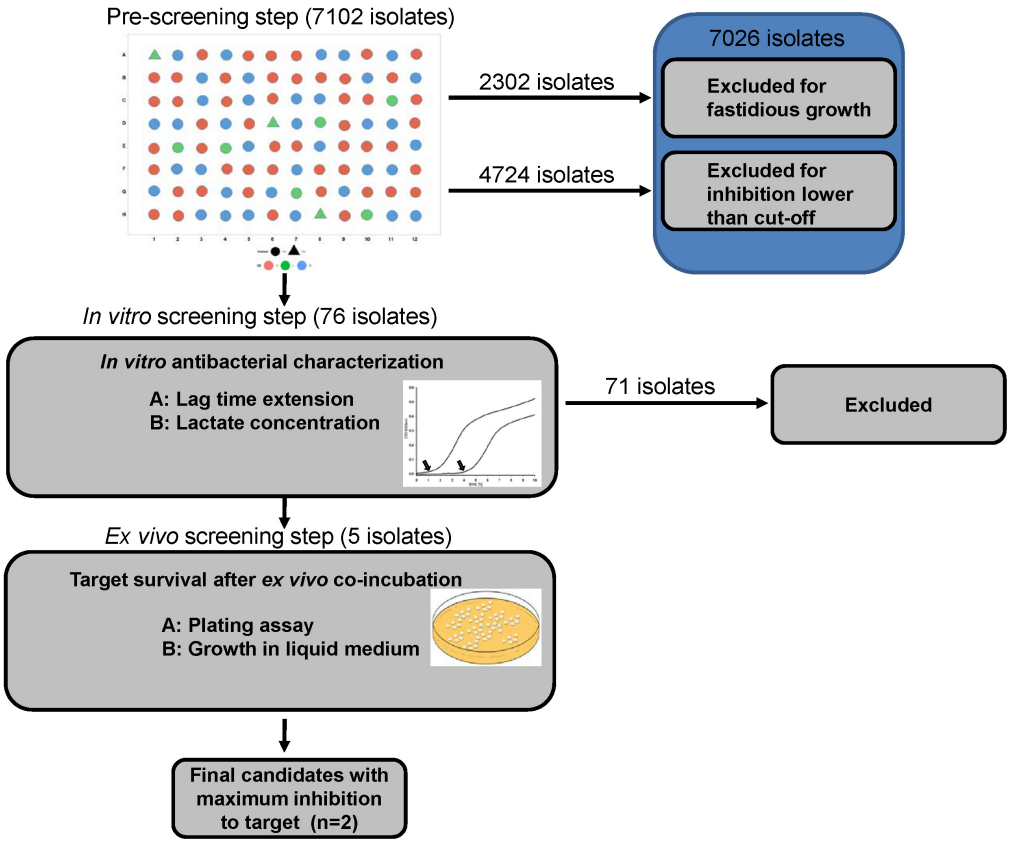

FIGURE 1 | Experimental design of the assay.

incubation at 37°C for 1.5 h, non-adhering bacteria were washed three times with PBS. The monolayer of cells was detached with cell scratcher and re-suspended with 500 μl PBS. After a serial dilution, detached cells were then plated onto MRS agar plates or BHI agar plates supplemented with 8 μg/ml cefotaxime. Adhesion and competitive adhesion of lactobacilli was determined by enumeration of colonies on agar plates and calculated as relative to controls.

Antibiotic Susceptibility

The minimum inhibitory concentration (MIC) of a selected panel of antibiotics including ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, and tetracycline toward candidates were determined using a broth microdilution test as described by the Clinical and Laboratory Standards Institute (CLSI) with minor modification (CLSI, 2012). Selected candidates were incubated as described. Microdilution plates containing 100 μl MRS medium were inoculated with 50 μl inoculum as well as 50 μl antibiotic solution at appropriate concentration (0.25–128 μg/ml). Negative and positive controls were non-inoculated/inoculated wells without antibiotics. After anaerobic incubation at 37°C for 48 h, the MICs were determined as their lowest concentration capable to inhibit the visible bacterial growth. The reference strain DSM 20016 (*L. reuteri*) was used as the quality control. The cut-off value documented by European Food Safety Authority (EFSA, 2012) was used to categorize susceptibility or resistance of selected candidates.

Statistical Analysis

The experiments were performed twice in triplicates for the determination and comparison in screening and characterization section. Results are presented as means ± standard deviation (SD). For *in vitro* data, lag times were modeled and analyzed by 3-parameter sigmoidal equation using SigmaPlot version 11 (Systat Software Inc., United States). Statistical significance of comparison in screening steps was assessed using Mann–Whitney test. Significance of different cell adhesion level was evaluated with Duncan's multiple range test. Statistical procedures were performed at a significance level of 95%. All calculations were performed using the statistics software IBM SPSS (Version 22, Chicago, IL, United States).

RESULTS

Step 1: Massive Isolation and Preliminary Screening of Intestinal Lactobacilli

In the initial screening step, 7102 colonies were processed. 2302 isolates failed to show growth after sub-culturing colonies

in liquid medium. The remaining 4800 isolates were further tested for inhibitory activity against the model *E. coli* strain with the described buffering or deacidification treatments. Of those isolates, a total of 76 isolates showed either strong growth inhibition ($OD_{690nm} < 0.2$) in buffered supernatants (48 of 2160 isolates tested, 2.2% positive) or in deacidified supernatants (28 of 2564 isolates tested, 1.1% positive).

Step 2: *In vitro* Selection

A more in-depth evaluation of the inhibitory activity of isolates was studied by monitoring *E. coli* lag time lag time after incubation in supernatants. The production of lactic acid by the isolates was used as an additional inhibitory parameter, as lactic acid is strongly inhibitory to most enterobacteria. Increase of lag time of the *E. coli* strain in supernatants ranged from 1.17 h to 2.57 h and lactic acid production in overnight cultures ranged from 14.07 g/L to 16.01 g/L (**Table 1**). From the comprehensive comparison of both lag time and lactic acid production indices, five isolates were chosen for the final step (**Figure 2**).

Detailed lag times of all 76 isolates were shown in **Supplementary Table 1**. Regarding the original selection, two of the five strains were isolated via the buffer system, while three strains were obtained from the deacidification treatment.

Step 3: *Ex vivo* Selection

In the *ex vivo* selection step, five isolates from the *in vitro* selection were co-incubated with the *E. coli* model strain in intestinal contents of broiler chicken. After 8 h co-incubation, most candidates showed a stronger inhibitory activity against the *E. coli* model strain in crop contents than in jejunum contents (**Figures 3A,B**). Strain S26 only led to reduced growth of the *E. coli* strain but all other strains resulted in complete inhibition in crop content. No inhibition by all strains was observed after 8 h in jejunum contents. When the *ex vivo* co-incubation was extended to 24 h and studied via CFU, the inhibitory effects of the candidate isolates were amplified (**Table 2**). These results indicate that candidate S1 and S73 completely reduced the survival of *E. coli* in intestinal contents.

Characterization of Final Lactic Acid Bacteria Candidates

Data on the characterization of the Lactobacillus isolates is shown in **Table 3**. The final 2 candidates, *Lactobacillus* strains S1 (*L. salivarius*) and S73 (*L. agilis*) originated from ileum and crop samples of 42-day old broilers, respectively. Strain S1 was found using the buffer system, while S73 originated from the deacidification treatment. Strain S73 exhibited a stronger production of total SCFA in MRS medium than strain S1 (the production of lactic acid was included in **Table 1**). Regarding auto-aggregation, S73 showed a higher rate than S1. As to co-aggregation, no significant difference was observed in co-aggregative ability with the indicator *E. coli* strain after 24 h incubation. All candidates demonstrated good surface aÿnity and S73 revealed maximum hydrophobicity.

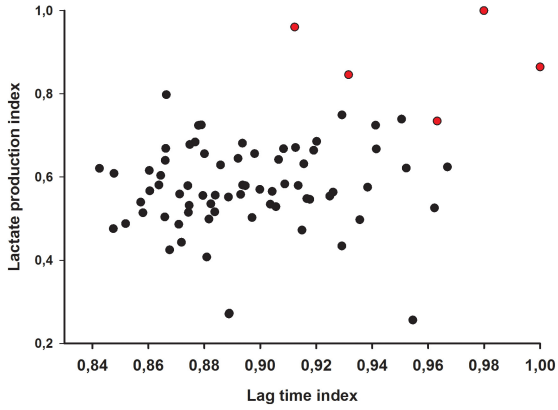

FIGURE 2 | Lag time and lactic acid index of 76 lactic acid bacteria isolates. Red dots indicate selected candidate isolates.

TABLE 1 | Lactic acid production of five lactic acid bacteria candidates and lag time of the *E. coli* model strain in media supplemented with supernatants of the candidates.

Strain	Lactic acid (g/L)	Lag time (h)
S1	15.06 ± 1.96	8.57 ± 1.16*
S26	14.07 ± 4.35	8.01 ± 0.79
S62	15.30 ± 2.65	8.09 ± 0.79
S70	16.01 ± 3.08	8.69 ± 0.83*
S73	15.46 ± 3.01	8.86 ± 1.39*
Control	–	6.29 ± 0.87

* = *Significantly different to control ($p \leq 0.05$, Mann–Whitney test).*

Evaluation of Stress Tolerance

Tolerance against gastric pH conditions and small intestinal bile acids was tested to study the survival of the isolates during their passage through stomach and small intestine. Growth of both candidates was suppressed at pH 2, but survival increased at pH 3–4 (**Table 4**). Strain S73 seemed to tolerate lower pH slightly better than S1.

Both strains survived bile acid supplemented media well in the range from 2.45 to 7.35 mM (0.1% to 0.3% w/v) bile concentration, while 17.15 mM (0.7% w/v) concentration of bile exhibited stronger inhibitory effects (see **Table 4**). However, S1 generally showed slightly reduced tolerance in bile supplemented MRS medium compared to S73.

Both candidates demonstrated good resistance against increasing osmolarity (**Table 5**). Growth could still be detected until 8% NaCl. S1 showed a slightly better osmolarity resistance compared to S73.

Antibiotic Susceptibility

The results in MIC test of selected candidates were interpreted according to the "Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance" documented by ESFA (2012). No resistance was observed against ampicillin, clindamycin, streptomycin and tetracycline. The strain S26 and S62 demonstrated resistance against gentamycin and kanamycin. S26 also indicated the

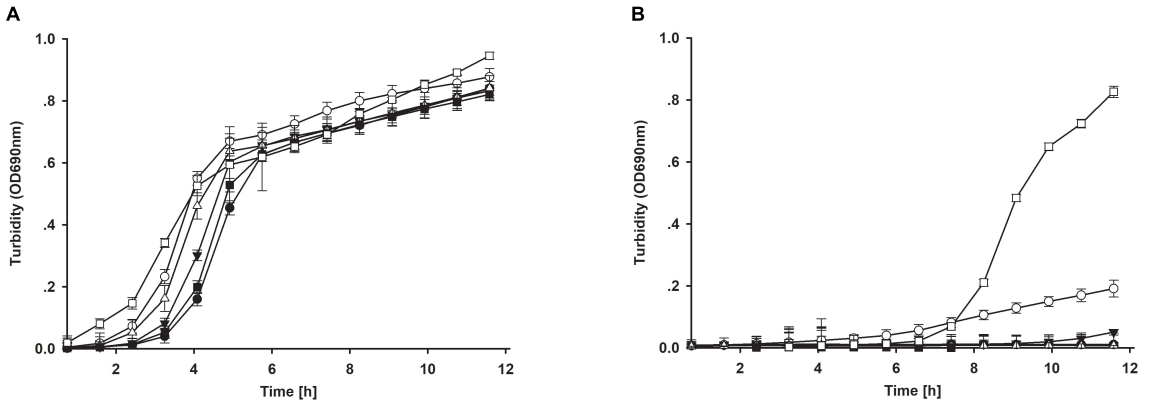

FIGURE 3 | (A) Growth of the model *E. coli* strain after *ex vivo* co-incubation with candidate lactic acid bacteria isolates for 8 h in crop contents. Filled circle = S1; open circle = S26; filled down triangle = S62; open downward triangle open diamond = S70; filled square = S73; open square = control. **(B)** Growth of the model *E. coli* strain after *ex vivo* co-incubation with candidate lactic acid bacteria isolates for 8 h in jejunum contents. Filled circle = S1; open circle = S26; filled down triangle = S62; open downward triangle open diamond = S70; filled square = S73; open square = control.

TABLE 2 | Survival of the *E. coli* model strain after 24 h incubation with lactic acid bacteria candidates in intestinal contents (CFU/g content).

	Crop	Jejunum
S1	ND	ND
S26	$7.40 \pm 0.67 \times 10^3$	$4.00 \pm 0.54 \times 10^4$
S62	$3.10 \pm 0.50 \times 10^3$	$6.80 \pm 0.42 \times 10^3$
S70	$7.20 \pm 0.80 \times 10^2$	$3.80 \pm 0.54 \times 10^2$
S73	ND	ND
E. coli control	$3.80 \pm 0.22 \times 10^4$	$9.10 \pm 1.79 \times 10^4$
Initial *E. coli* count	$8.70 \pm 1.35 \times 10^4$	$8.70 \pm 1.35 \times 10^4$
Negative control	ND	ND

ND = not detected (detection limit: 10^2 CFU/g content).

resistance to chloramphenicol and erythromycin. S70 showed the resistance to kanamycin. The maximum susceptibility was observed against ampicillin and clindamycin. As the breakpoint of cefotaxime was not included in the documentation of ESFA, the results only revealed none of candidates was resistant to the cefotaxime at working concentration (8 μg/mL) of *ex vivo* model (**Table 6**).

Adhesion and Competitive Adhesion Assay

Among the five candidates tested in *ex vivo* model, S1 demonstrated the best adhesion capacity to human Caco-2 cell lines (**Figure 4A**). The competitive adhesion assay showed that the adhesion of *E. coli* model strain decreased significantly when co-incubated with all lactobacilli candidates except with strain S26 (**Figure 4B**).

DISCUSSION

Benefits of probiotics in animal nutrition are increasingly highlighted for their improvement of animal health by reducing the pathogenic bacterial load and the increase in animal performance (feed conversion, body weight gain) (Hong et al., 2005; Taras et al., 2005; Böhmer et al., 2006). Contrary to probiotics in human medicine/nutrition, probiotics in animal nutrition are often expected to specifically combat pathogenic bacteria (Markowiak and Śliżewska, 2018) which are of major concern in farm animal husbandry. Therefore, the selection of probiotics against those veterinary pathogens is always the preferred solution to improve animal health. In our current study, a combination of *in vitro* and *ex vivo* method is introduced to enable a rapid and comprehensive selection selection from massive probiotic LAB that are active against *E. coli*.

The scientific rationale for the focus on host specific lactobacilli as potential probiotics in this study is based on following reasons. Firstly, lactobacilli are known for their antagonistic activity against *E. coli* (Juven et al., 1991; Servin, 2004; Arena et al., 2018). Secondly, lactobacilli enjoy the generally-regarded-as-safe (GRAS) status as defined by the FAO or qualified presumption of safety (QPS) in the EU. Thirdly, several studies indicate that bacteria are expected with higher chance to colonize their hosts, if they are isolated from the same host (Yuki et al., 2000; Kwong et al., 2014). Thus, choosing lactobacilli as main target of isolation, the functional criterion (inhibition of *E. coli*) was combined with safety considerations (GRAS/QPS status) and high probability of viability in the intestinal tract. As highlighted by the FAO, a major potential safety concern of LAB can be their antibiotic resistance. In our antibiotic susceptibility assay on the five isolates used for *ex vivo* selection step, both final candidates (strain S1 and S73) demonstrated no resistance against recommended antibiotics, which increases the confidence of their use as safe feed additive in the future. Finally, host specificity has been deemed a favorable property for probiotic microorganisms (Saarela et al., 2000). Consequently, the robustness of potential probiotics against specific conditions of the GIT should be a pre-requisite for any candidate strain planned for *in vivo* colonization (Dicks and Botes, 2010; Fiocco et al., 2019). As the LAB candidates in this

TABLE 3 | Characterization of lactic acid bacteria candidates.

Isolates	Sampling site	Morphology	Taxonomic identification	SCFA production (μmol/mL)	Auto-aggregation (%)	Co-aggregation (%)	Hydrophobicity (%)
S1	Ileum	Rod	*L. salivarius*	96.13	42.31 ± 2.49	35.30 ± 2.17	65.57 ± 2.83
S26	Feces	Spherical	*P. acidilactici*	104.57	43.33 ± 2.05	33.33 ± 1.56	38.73 ± 1.58
S62	Crop	Rod	*L. agilis*	92.41	46.57 ± 0.91	37.12 ± 1.68	49.10 ± 1.75
S70	Feces	Rod	*L. salivarius*	96.51	41.35 ± 2.34	36.45 ± 3.30	45.97 ± 3.70
S73	Crop	Rod	*L. agilis*	124.18	53.98 ± 2.93	34.79 ± 1.57	70.13 ± 2.27

TABLE 4 | Viability of final candidates under acidic conditions or bile challenge (log CFU/mL).

		Incubation time	S1	Survival percentage (cell) (%)	S73	Survival percentage (cell) (%)
Acid tolerance		0 h	8.14 ± 0.06	100	8.10 ± 0.042	100
	pH = 2	2 h	7.50 ± 0.00	22.91	7.86 ± 0.14	57.54
		4 h	5.77 ± 0.23	0.43	6.00 ± 0.20	0.79
		6 h	4.88 ± 0.09	0.05	5.22 ± 0.20	0.13
	pH = 3	2 h	7.70 ± 0.18	36.31	7.93 ± 0.07	67.61
		4 h	6.27 ± 0.05	1.35	7.21 ± 0.20	12.88
		6 h	6.01 ± 0.00	0.74	6.40 ± 0.01	2.00
	pH = 4	2 h	7.96 ± 0.25	66.07	7.99 ± 0.22	77.62
		4 h	7.07 ± 0.14	8.51	7.67 ± 0.13	37.15
		6 h	6.36 ± 0.01	1.66	6.86 ± 0.00	5.75
Bile tolerance		0 h	7.63 ± 0.07	100	8.02 ± 0.09	100
	2.45 mM	2 h	7.46 ± 0.05	20.89	7.60 ± 0.10	31.62
		4 h	6.69 ± 0.07	3.55	6.91 ± 0.03	6.46
		6 h	6.49 ± 0.07	2.24	6.74 ± 0.07	4.37
	7.35 mM	2 h	7.06 ± 0.03	8.32	7.31 ± 0.10	16.22
		4 h	6.71 ± 0.04	3.72	6.53 ± 0.05	2.69
		6 h	6.31 ± 0.08	1.48	6.39 ± 0.15	1.95
	12.25 mM	2 h	6.83 ± 0.01	4.90	6.850 ± 0.03	5.62
		4 h	6.03 ± 0.11	0.78	6.30 ± 0.07	1.58
		6 h	5.61 ± 0.12	0.30	5.83 ± 0.15	0.54
	17.15 mM	2 h	6.54 ± 0.08	2.51	6.70 ± 0.13	3.98
		4 h	5.93 ± 0.16	0.62	6.08 ± 0.05	0.95
		6 h	5.26 ± 0.12	0.13	5.69 ± 0.07	0.39

TABLE 5 | Growth capacity of final candidates under different osmotic pressures (final OD_{690nm}).

	0% NaCl	2% NaCl	4% NaCl	6% NaCl	8% NaCl	10% NaCl
S1	1.12 ± 0.09	0.99 ± 0.03	0.85 ± 0.01	0.60 ± 0.01*	0.52 ± 0.06*	0.15 ± 0.02
S73	0.99 ± 0.11	0.82 ± 0.08	0.70 ± 0.07	0.34 ± 0.03	0.26 ± 0.05	0.13 ± 0.06

* = *Significantly different between strains ($p \leq 0.05$. Mann–Whitney test).*

study were specifically designated to be used in broiler chicken, we followed this host-specific concept. To further ascertain the host specificity, an incubation temperature of 39°C has been applied to simulate the body temperature of broiler chicken and consequently yield mostly host-specific LAB isolates. In future applications of this method, this parameter can be changed depending on the host of interest.

The novelty in our procedure firstly lies in the number of screened isolates because enlarging the sample number simply enhances the chance to find probiotic isolates. Secondly, the validation of an *ex vivo* screening based on the survival of the model strain co-incubated with candidate LAB of interest under simulated *in vivo* conditions possibly predicts their functional activity in host animal. Thus, our experimental design combines three consecutive steps to progressively reduce the number of candidates with multiple criteria step by step (de Melo Pereira et al., 2018).

A literature search on isolation of probiotic bacteria revealed that the number of isolates ranges from 14 to 1150 isolates with the majority of publications using only 50 to 80 isolates (Robyn et al., 2012; Babot et al., 2014). To increase the number of potential isolates, a procedure allows both high throughput and easy method for detection of inhibitory activity is needed. Therefore, we designed a pre-screening step to identify potential candidates out of a massive sample pool (over 7000 isolates) by systematic processing

TABLE 6 | Susceptibility test of selected candidate strains to antibiotics.

	S1	S26	S62	S70	S73	QC strain
Ampicillin	$<0.25^S$	2^S	$<0.25^S$	$<0.25^S$	0.25^S	0.5^S
Chloramphenicol	4^S	8^R	4^S	4^S	2^S	2^S
Clindamycin	0.5^S	$<0.25^S$	0.25^S	1^S	1^S	$<0.25^S$
Erythromycin	0.25^S	2^R	0.25^S	1^S	1^S	1^S
Gentamycin	16^S	64^R	64^R	8^S	16^S	8^S
Kanamycin	32^S	128^R	128^R	128^R	32^S	16^S
Streptomycin	32^S	32^S	16^S	8^S	16^S	16^S
Tetracycline	4^S	2^S	4^S	1^S	4^S	2^S
Cefotaxime	0.25	2	2	1	1	2

S = susceptible; R = resistant; QC strain = L. reuteri (DSM20016).

instead of one-by-one treatment. This procedure also identified isolates which exhibit ease of growth and handling as a prerequisite for production of probiotics on a technical scale. Elimination of LAB that could not be cultivated under the relatively simple growth conditions of the assay probably led to a loss of many strains with potential inhibitory activity. However, fastidious growth conditions will inevitably lead to prohibitively high costs during later biomass production and thus, commercialization of the obtained isolates would be questionable.

During the selection progress, buffered or deacidified supernatants were used. It is known that lactic acid produced by lactobacilli can drastically reduce pH in media. Therefore, buffered/deacidified supernatants exclude pH-dependent inhibition via metabolites except for exceptionally high lactic acid production that overcomes the buffering capacity. On the other hand, a pH-independent inhibition involves different modes of action like production of bacteriocin or bacteriocin-like-substances (BLIS). In the present assay, both pH-dependent and pH-independent modes of action were considered.

The pre-screening step yielded 76 potential probiotic isolates out of the initial 4800 robust isolates (1.6%). These isolates were characterized in more depth via lag time extension of the *E. coli* model strain and lactic acid production. Since lag time extension is a key indicator in evaluating growth inhibition of microorganisms under adverse conditions (Swinnen et al., 2004; Rufián-Henares and Morales, 2008), it is often used to assess growth inhibition to various target microorganisms (Pereira et al., 2016; Alpaslan et al., 2017). The advantage of liquid based growth inhibition assays over agar diffusion assays is their sensitivity to observe subtle influences on growth (Fredua-Agyeman et al., 2017), and also their sample throughput. Therefore, we chose a microtiter plate-based assay to fit the purpose of rapid and eÿcient screening potentially probiotic LAB. Lactic acid production was considered as another inhibitory parameter, because exceptional lactic acid production *in vitro* may also yield high lactic acid production *in vivo*. For the studied isolates, it was also shown *in vitro* that other metabolites such as short chain fatty acids are negligible compared to lactic acid. The classic antagonism requires lactic acid to acidify the environment, which in turn inhibits growth of non-acid fast bacteria. However, lactic acid also exerts additional inhibition by disrupting the outer membrane to Gram-negative bacteria including *E. coli* (Alakomi et al., 2000). The use of lag time and lactic acid production indices enables the identification of isolates with the highest inhibitory activities. In the end, we chose five isolates among all candidates that exhibited the highest indices for both parameters.

In view of the complex environment in the intestinal tract, *in vitro* models cannot reflect antibacterial effects that may occur

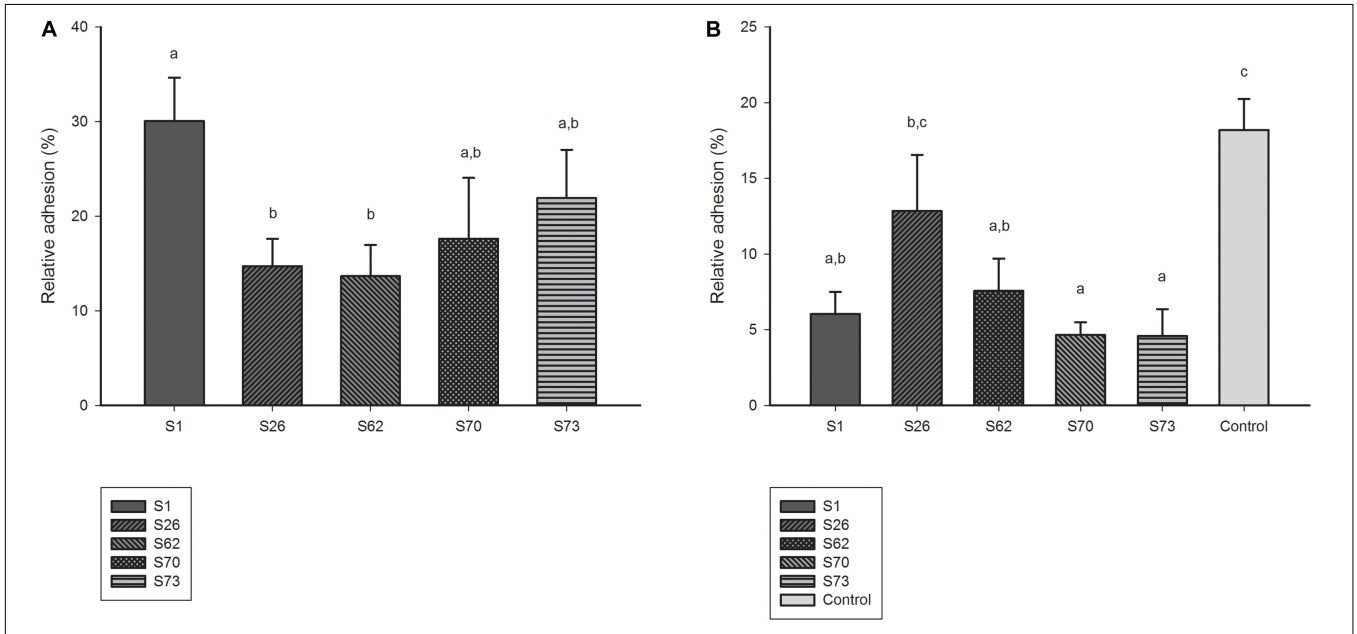

FIGURE 4 | (A) Adhesion ability of lactobacilli candidates to Caco-2 cell monolayers. **(B)** Adhesion ability of model *E. coli* strain co-incubated with lactobacilli candidates to Caco-2 cell monolayers.

in the animal. It is always questionable whether probiotics inhabit or maintain their inhibitory activity *in vivo* well (Talpur et al., 2012). Consequently, *in vitro* tests are not able to mimic the complex intestinal matrix and truly reflect the inhibitory activity of probiotics in the GIT of animal (Saint-Cyr et al., 2016). However, *in vivo* experiments are costly and are subject to ethical considerations. *Ex vivo* assays try to find a compromise between both approaches. *Ex vivo* assays are advantageous due to higher replicate numbers and application of biological agents at defined concentrations. In the present study, although a two-fold dilution of digesta content may have led to a bias regarding the response of the biological matrix (partly hydrolyzed nutrients, metabolites, etc.). Nevertheless, part of the biological matrix was still intact and previous studies have shown that this *ex vivo* assay has the potential to mimic the bacterial response in intestinal contents. For instance, Starke et al. (2014) used a very similar *ex vivo* system on the response of intestinal bacteria to zinc and found that the system correctly predicted the bacterial response to zinc of later pig trials (Starke et al., 2014). Therefore, although the chosen *ex vivo* assay in this study had its limits, it still is a valuable tool to more closely elucidate possible inhibitory activity of bacterial isolates *in vitro*.

Compared to *in vitro* assays, the tested *Lactobacillus* candidates demonstrated different inhibitory effects against the *E. coli* model strain in the *ex vivo* model. Here, candidate S1 (*L. salivarius*) and S73 (*L. agilis*) showed the highest inhibitory potential. The other chosen LAB strains were not able to completely inhibit *E. coli* growth, although their *in vitro* performance was superior. Thus, the *ex vivo* assay has shown that it was indeed worthwhile to use an intermediate step before using probiotic isolates directly in feeding trials.

As recommended by WHO for selecting probiotics, host-related stress tolerance is usually considered as screening criteria in many studies (de Melo Pereira et al., 2018). The GIT induced stress was simulated *in vitro* according previous publications (Mongin et al., 1976; Lin et al., 2003; Lemme and Mitchell, 2008; Morgan et al., 2014; Nkukwana et al., 2015). Both *Lactobacillus* candidates demonstrated high viability in acidic incubations, maintained growth at pH 4, tolerated a wide range of bile concentrations and showed good resistance against high osmolality. Thus, a good survival in the GIT of the strains is expected and was predictable as they were isolated from the crop (S73) or ileum (S1) of broiler chicken. This also underscores the notion that host specific isolation increases the probability to isolate candidates with high survival rates in their respective host. However, four of the studied *Lactobacillus* isolates also inhibited *E. coli* adhesion in a commonly used intestinal model cell line, the Caco-2 cell lines, which may indicate a potential benefit of the selected candidates in competitive actions for intestinal niche. Metabolite production was also monitored and as expected, only minor amounts of acetate was found compared to production of lactic acid, while only traces of propionate and butyrate were present. This is in agreement with some previous studies (Imen et al., 2015). The level of propionate, butyrate and valerate was relatively low. This phenomenon might be because of being consumed as the energy for bacterial survival (Fernando et al., 2018).

The final two *Lactobacillus* spp. are currently used in feeding trials. Preliminary results indicate that the strains indeed modified the bacterial composition and activity metabolite concentration in the intestinal tract of broiler chicken (data not shown). Eventually, the employed combination of *in vitro* and *in vivo* combined method has the potential to isolate other probiotic bacteria with inhibitory activity against any other specific bacterium, as long as a specific biomarker for pathogens (for instance antibiotic resistance) is available. With modification regarding growth condition as well as the detection method for the bacterium in question, the described method can be expanded to other probiotic species for a targeted search against specific microbes. This gives the method a general applicability in a more comprehensive and rapid way.

CONCLUSION

In the present study, a novel three-strep rapid screening method consisted is reported for the isolation of probiotic LAB against a target *E. coli*. It includes a pre-screening step as an effective filter of a massive isolate pool and easy-handling of the isolates for later technical scale cultivation; an *in vitro* selection step to assure the correct choice of the most active isolates and finally, an *ex vivo* assay to confirm probiotic function of the candidates *in vivo*. As a proof-of-principle we have chosen lactobacilli as antagonist to *E. coli*, but the system can be employed to screen any cultivable probiotic bacterium and its inhibitory activity against any cultivable bacterium with a specific biomarker.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Ethical approval Samples were taken from studies that were conducted in accordance with the German Animal Welfare Act (TierSchG) and approved by the local state oÿce of occupational health and technical safety "Landesamt für Gesundheit und Soziales, Berlin" (LaGeSo Reg. Nr. T 0162/16 and A 0100/13).

AUTHOR CONTRIBUTIONS

HR, WV, and JZ organized the whole study. HR and WV developed the protocol of the screening method. HR performed the experiments, analyzed data and wrote the first version of the manuscript. E-MS contributed to the selection of target *E.coli* and provided the basic information about the bacteria. FG contributed to the animal trial and sampling. WV, JZ, E-MS, and FG revised the manuscript. All authors read and approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02705/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 5: Optimization of Production Parameters for Probiotic *Lactobacillus* Strains as Feed Additive

Hao Ren*, Jürgen Zentek and Wilfried Vahjen

Institute of Animal Nutrition, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany.
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*Correspondence: hao.ren@fu-berlin.de; Tel.: +49 30 83850149

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Optimization of Production Parameters for Probiotic Lactobacillus Strains as Feed Additive

Hao Ren *, Jürgen Zentek and Wilfried Vahjen

Institute of Animal Nutrition, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany; Juergen.Zentek@fu-berlin.de (J.Z.); wilfried.vahjen@fu-berlin.de (W.V.)

* Correspondence: hao.ren@fu-berlin.de; Tel.: +49-30-83850149

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Abstract: In animal nutrition, probiotics are considered as desirable alternatives to antibiotic growth promoters. The beneficial effects of probiotics primarily depend on their viability in feed, which demands technical optimization of biomass production, since processing and storage capacities are often strain-specific. In this study, we optimized the production parameters for two broiler-derived probiotic lactobacilli (*L. salivarius* and *L. agilis*). Carbohydrate utilization of both strains was determined and preferred substrates that boosted biomass production in lab-scale fermentations were selected. The strains showed good aerobic tolerance, which resulted in easier scale-up production. For the freeze-drying process, the response surface methodology was applied to optimize the composition of cryoprotective media. A quadratic polynomial model was built to study three protective factors (skim milk, sucrose, and trehalose) and to predict the optimal working conditions for maximum viability. The optimal combination of protectants was 0.14g/mL skim milk/ 0.08 g/mL sucrose/ 0.09 g/mL trehalose (*L. salivarius*) and 0.15g/mL skim milk/ 0.08 g/mL sucrose/ 0.07 g/mL (*L. agilis*), respectively. Furthermore, the in-feed stabilities of the probiotic strains were evaluated under different conditions. Our results indicate that the chosen protectants exerted an extensive protection on strains during the storage. Although only storage of the strains at 4 °C retained the maximum stability of both *Lactobacillus* strains, the employed protectant matrix showed promising results at room temperature.

Keywords: probiotic; optimization procedure; freeze-drying; response surface method; in-feed stability

1. Introduction

The development of alternatives for antibiotic growth promoters in livestock has been of global interest in the field of animal nutrition after their ban in many countries [1–3]. Probiotics have shown beneficial effects in the field of animal nutrition such as improved weight gain, development of a beneficial intestinal microbiota, and enhancement of the immune system in farm animals [4–6]. Most probiotic are bacteria, but there are also few non-bacteria microorganisms like yeast that belong to the probiotic family [7]. As an important member of lactic acid bacteria, *Lactobacillus* has become one of the most commonly used probiotic species among all probiotic species [8]. Health promotion by lactobacilli, which are generally regarded as safe (GRAS), makes them particularly interesting as a zootechnical additive [9,10].

A sufficient number of viable probiotic cells is a prerequisite for their successful impact in the animal [11]. In order to commercialize probiotics, timesaving and cost-effective methods to increase bacterial cell yield during the production progress are necessary [12]. Among other parameters, biomass production can be improved by adjusting growth factors (e.g., substrates, pH, incubation time) to optimize biomass production [13–15]. Another fundamental factor is the cost intensive fermentation especially of anaerobes, which negatively affects the scale-up of biomass production [16]. This topic has been investigated in several studies, but with a limited number of candidate species [17].

The preparation of probiotic products calls for reasonable cell stability during the manufacturing process. Among various techniques, drying methods are commonly used for the preservation and ease of handling of microorganisms [18]. Freeze-drying has been widely applied to bacteria that exhibit high stability against low temperatures [19]. However, stress factors such as very low freezing temperatures or dehydration during freeze-drying can cause undesirable loss of viability for some probiotic strains [20,21]. Due to this, a variety of cryo-protectants have been developed to increase the viability of probiotic bacteria during the freeze-drying procedure [22,23]. Protectants such as skim milk, whey proteins, sugars, or other bio-polymers were studied mostly as combinations for synergistic protective effects with other protectants [24,25]. The classical one-variable-at-a-time approach (OVAT) strategy was deemed more time-consuming. It ignores the interaction between functioning factors, which might lead to the confusion and bias of results [26]. Thus, the response surface method has become one of the most used optimization approaches to create the best conditions with a minimum number of experiments [25]. Among different optimization procedures, the Box-Behnken Design (BBD) has been shown to be superior to 3-level full factorial designs and is, thus, being used in response surface modelling [27,28]. Furthermore, results indicated that cryo-protectants might work in a strain-specific manner and, thus, optimization may rely on particular protective systems for a given strain.

Viability and activity of probiotics during storage are critical criteria for both the manufacturer and customer [29]. Storage conditions affect survival of bacterial cells [30] and can even influence the functionality of the probiotic such as stress resistance or capacity of epithelial adhesion without changing cell viability [31]. Most studies report on the storage stability of probiotics as a sole objective. However, in-feed stability is of prime importance, but is seldom reported.

In a previous study, two *Lactobacillus* strains (*L. salivarius*, *L. agilis*) were isolated from broiler intestinal samples (unpublished data). These strains were tested for their applicability as a probiotic additive for poultry. The current study determined the most economical and feasible procedure to produce those probiotic strains as feed additive. Furthermore, different factors regarding biomass production, survival during lyophilization, and in-feed stability of storage were evaluated.

2. Results

2.1. Metabolic Fingerprints of the Lactobacillus Strains

The results for the BIOLOG® AN plates are shown as a heat map in Figure 1. The *L. salivarius* strain showed a broader carbohydrate utilization spectrum than the *L. agilis* strain. The highest metabolic activity for the *L. salivarius* strain was observed for maltose, raffinose, sucrose, and glucose, while the *L. agilis* strain metabolized mannose, glucose, L-lactic acid, and mannitol as preferred carbohydrate substrates followed by mannitol, lactic acid, mannose, glucose, maltose, sucrose, maltotriose, lactose, melibiose, raffinose, sorbitol, and lactulose. Taking cost and easy-availability of those substrates into consideration, mannitol, mannose, maltose, sucrose, melibiose, and sorbitol were selected for further evaluation of boosting effects on lactobacilli growth.

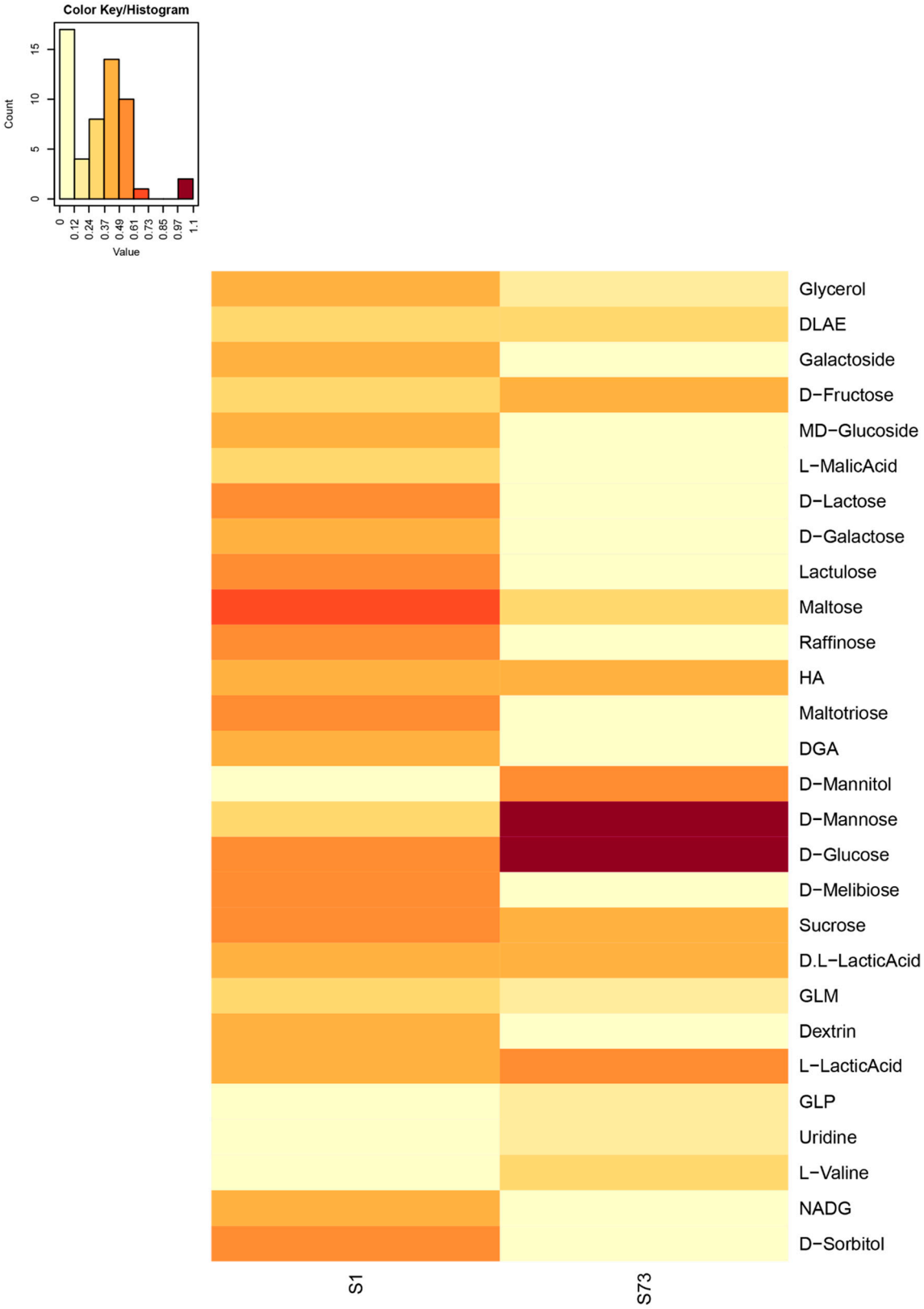

Figure 1. Metabolic fingerprint of the probiotic *Lactobacillus* strains. DLAE = D-Lactic Acid Methyl Ester. HA = α- Hydroxybutyric Acid. DGA = D-Galacturonic Acid. GLM = Glycyl-L-Methionine. GLP = Glycyl-L-Proline. NADG = N-Acetyl-D-Glucosamine. S1 = *L. salivarius*. S73 = *L.agilis*.

2.2. Booster Effects of Selective Carbon Sources on Biomass Production

The addition of sucrose and sorbitol to the basal medium led to a significantly increased number of viable cells for strain *L. salivarius* after 12 h of incubation, while the addition of mannose revealed a booster effect on bacterial growth for the strain *L. agilis* (Table 1). Extension of cultivation time to 24 h showed that, all incubations exhibited lower viable cell numbers than after 12 h, except for incubations in the basal medium. The lowest viable cell numbers were observed after 48 h of incubation (see Table 1), whereas the biomass in all experimental groups decreased to a level significantly lower than in the MRS medium.

When comparing all cultivation situations, the incubation of 12 h with the addition of sucrose significantly increased the biomass yield of strain *L. salivarius* ($p = 0.05$). Although the addition of mannose did not significantly increase the biomass yield of *L. agilis* ($p = 0.127$), it ascertained that shortening the cultivation time still yielded high biomass for both strains. These two substrates were used in further tests to increase the biomass yield for *L. salivarius* and *L. agilis*, respectively.

Table 1. Biomass of the probiotic strains in media supplemented with different additional substrates at different time points [log CFU/mL].

	12 h		24 h		48 h	
	L. salivarius	***L. agilis***	***L. salivarius***	***L. agilis***	***L. salivarius***	***L. agilis***
Sucrose	9.22 ± 0.02 *	9.08 ± 0.02 *	8.94 ± 0.05 *	8.82 ± 0.06 *	8.67 ± 0.12	8.11 ± 0.06 *
Maltose	9.08 ± 0.05 *	9.11 ± 0.07	8.74 ± 0.12 *	8.74 ± 0.12 *	8.26 ± 0.13 *	8.1 ± 0.12 *
Mannitol	9.04 ± 0.11	9.02 ± 0.09	8.75 ± 0.19 *	8.54 ± 0.07 *	8.45 ± 0.09 *	8.21 ± 0.09 *
Mannose	9.1 ± 0.04 *	9.2 ± 0.06 *	8.61 ± 0.03 *	8.92 ± 0.05 *	8.49 ± 0.04 *	8.42 ± 0.1 *
Sorbitol	9.18 ± 0.06 *	9.17 ± 0.03 *	8.88 ± 0.03 *	8.88 ± 0.04	8.48 ± 0.11 *	8.22 ± 0.06 *
Melibiose	9 ± 0.06	9.03 ± 0.03	8.96 ± 0.05	8.52 ± 0.04 *	8.63 ± 0.06	8.27 ± 0.1 *
MRS contol	8.86 ± 0.1	8.98 ± 0.04	9.07 ± 0.07	9.13 ± 0.02	8.65 ± 0.04	8.65 ± 0.07

* = $p < 0.05$. MRS control: control medium (de Man, Rogosa, and Sharpe medium).

2.3. Effect of Aerobic or Anaerobic Incubation on Biomass Production

The tolerance of both strains to oxygen was evaluated by growth under aerobic or anaerobic conditions. Compared to aerobic conditions, the *L. salivarius* strain demonstrated numerically increased biomass under anaerobic conditions (11.97 ± 11.40 log CFU/L anaerobic vs. 11.90 ± 10.74 log CFU/L aerobic). There was also no significant difference in the biomass of strain *L. agilis* between anaerobic incubation and aerobic incubation (12.01 ± 11.17 log CFU/L anaerobic vs. 12.02 ± 11.07 log CFU/L aerobic).

2.4. Lyophilization and Optimization of Lyo-Protectants

With the purpose of defining the best survival of the strains after lyophilization, a total of 17 experiments with appropriate combinations of the three chosen protectants (skim milk, sucrose, and trehalose) were performed, according to the Box-Behnken Design (BBD).

Both actual and predicted responses of the strains with a different combination of factors were used for the establishment of a quadratic model (Supplemental Table S3). The ANOVA (Analysis of variance) fitted quadratic polynomial model is presented in Table 2. Data in both models were different with a high significance. The value of the determination coefficient also confirmed the goodness of fit for the polynomial model. Coefficients are the effects of each factor. By interpreting the results, it is possible to define the factor or factor combinations that have higher influence. The significances of all coefficients are shown in Table 2. In the current case, most linear coefficients, square coefficients, and interaction coefficients of the *L. salivarius* model (X_1, X_2, X_1X_2, X_2X_3, X_12, $X_2{}^2$, and $X_3{}^2$) and the *L. agilis* model (X_1, X_2, X_3, X_1X_2, X_1X_3, $X_2{}^2$, and $X_3{}^2$) were significant model terms, which confirmed the validation of the model.

Table 2. Coefficient estimates and ANOVA (Analysis of variance) analysis of the quadratic model for lactobacilli survival during the lyophilization process.

	Variables	Coefficient Estimates (± Standard Error)	F-Value	*p* Value	Model Significance	R^2
L. salivarius	Intercept	72.9 ± 0.4	233.22	<0.0001	<0.0001 **	0.9924
	Skim milk	6.64 ± 0.32	430.95	<0.0001		
	X_2	6.76 ± 0.32	446.4	<0.0001		
	X_3	0.59 ± 0.32	3.41	0.1071		
	Skim milk, sucrose	3.69 ± 0.45	66.4	<0.0001		
	X_1X_3	0.24 ± 0.45	0.29	0.6063		
	X_2X_3	2.11 ± 0.45	21.72	0.0023		
	X_1^2	−4.2 ± 0.44	90.93	<0.0001		
	X_2^2	−12.49 ± 0.44	802.54	<0.0001		
	X_3^2	−5.37 ± 0.44	148.45	<0.0001		
L. agilis	Intercept	77.26 ± 0.52	82.44	<0.0001	<0.0001 **	0.9786
	X_1	8.6 ± 0.41	440.12	<0.0001		
	X_2	3.19 ± 0.41	60.54	0.0001		
	X_3	2.37 ± 0.41	33.46	0.0007		
	X_1X_2	−1.79 ± 0.58	9.58	0.0174		
	X_1X_3	−1.47 ± 0.58	6.45	0.0387		
	X_2X_3	−1.06 ± 0.58	3.36	0.1095		
	X_1^2	−1.01 ± 0.56	3.23	0.1155		
	X_2^2	−6.07 ± 0.56	115.44	<0.0001		
	X_3^2	−4.23 ± 0.56	56.11	0.0001		

X_1 = skim milk. X_2 = sucrose. X_3 = trehalose. ** = $p < 0.01$.

The fitted response surface plots and their corresponding contour plots for the survival of the strains after lyophilization are shown in Figures 2 and 3. The diagnostic of the modelling demonstrated that all residuals of both responses were normally distributed as linearity, which validated the statistical assumption of the model (Supplementary Figure S1). The predicted vs. actual value of survival of both *L. salivarius* and *L. agilis* are presented in Figure 4.

The optimal concentration for each variable was deduced from the software as 0.14 g/L skim milk, 0.08 g/L and 0.09 g/L trehalose for *L. salivarius*, and 0.15 g/L skim milk, 0.08 g/L, and 0.07 g/L trehalose for *L. agilis*, respectively. With the optimized formulation of cryo-protectants, the maximum survival of both *L. salivarius* and *L. agilis* could be demonstrated (Table 3).

Table 3. Optimum process and validation experiment results at a 95% confidence interval.

Response Viability	Target	Predicted Results	Standard Deviation	95% PI Low	95% PI High
L. salivarius	Maximized	76.19	3.91	65.54	86.83
L. agilis	Maximized	84.77	1.16	81.56	87.97

PI = Prediction interval.

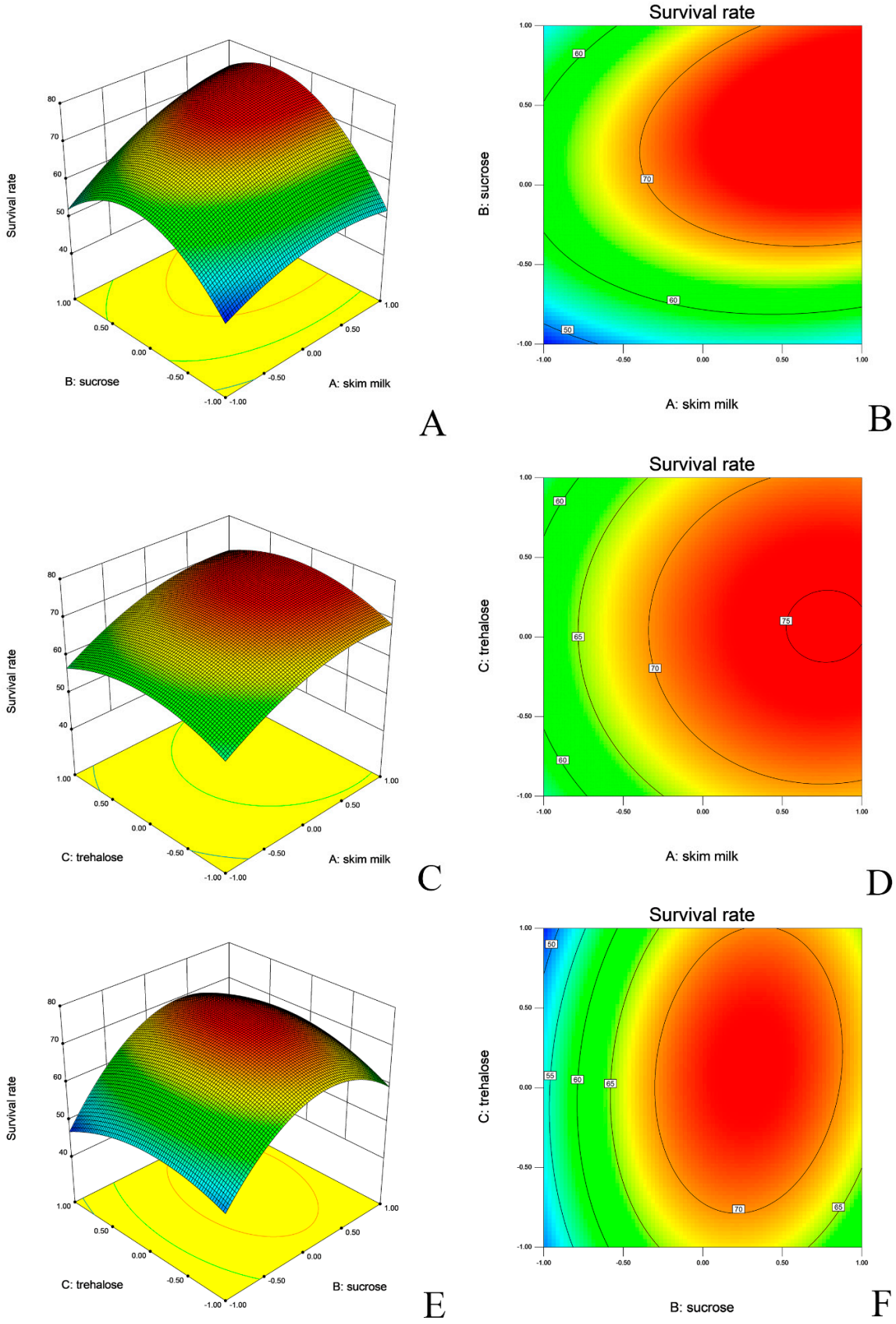

Figure 2. Response surface and contour plots depicting *L. salivarius* viability after lyophilization. (**A**,**B**): skim milk vs sucrose. (**C**,**D**): skim milk vs. trehalose. (**E**,**F**): sucrose vs. trehalose.

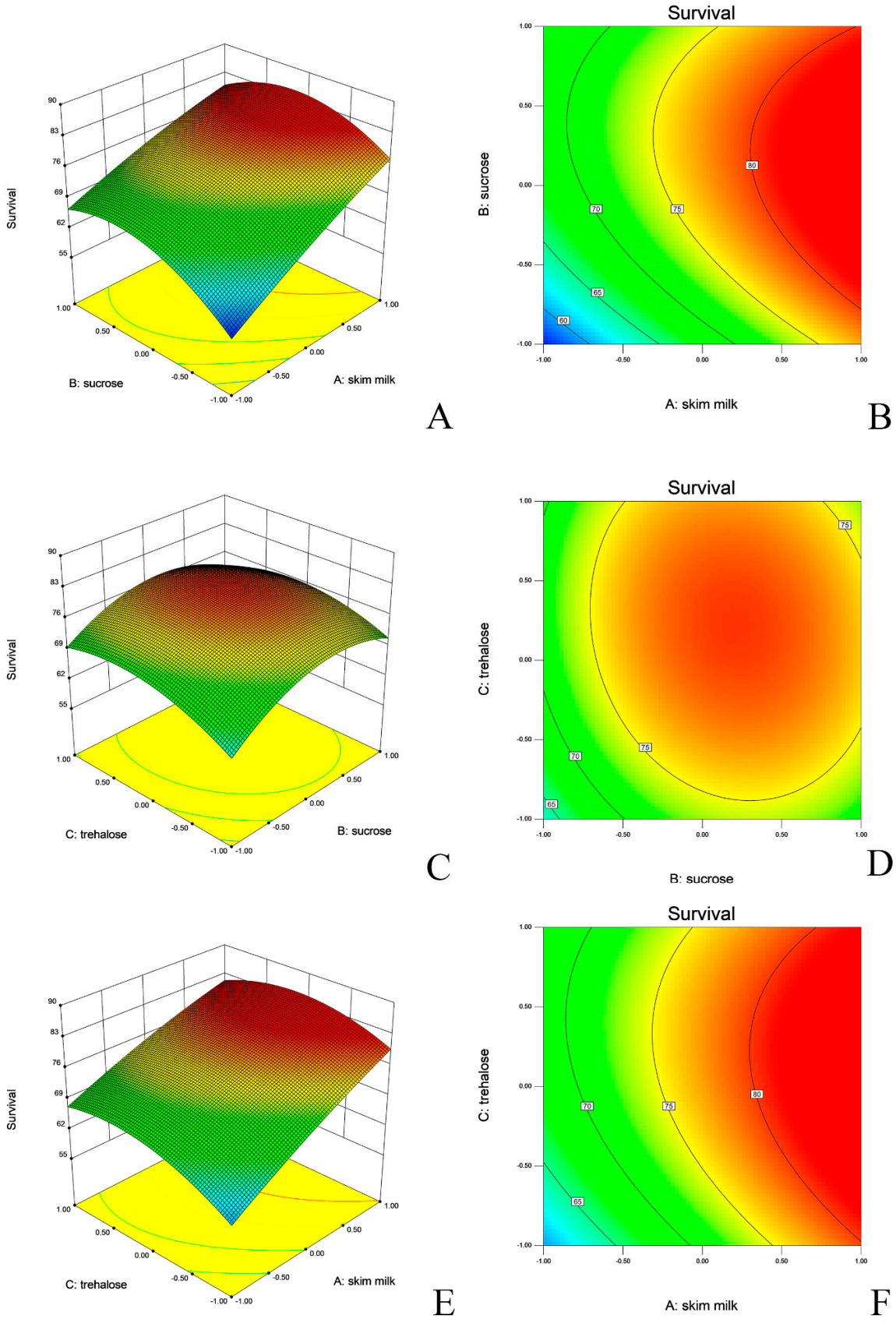

Figure 3. Response surface and contour plots depicting *L. agilis* viability after lyophilization. (**A**,**B**): skim milk vs. sucrose. (**C**,**D**): sucrose vs. trehalose. (**E**,**F**): skim milk vs. trehalose.

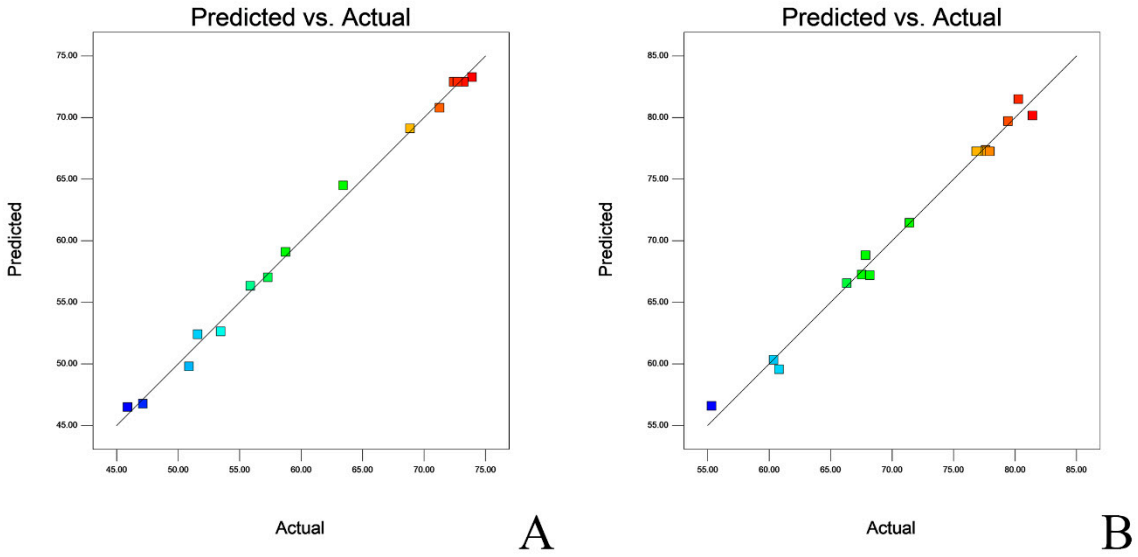

Figure 4. Linear plot fitting predicted vs. actual viability of lactobacilli. (**A**): *L. salivarius*. (**B**): *L. agilis*.

2.5. Stability during In-Feed Storage

The stability of both strains was determined according to their time-dependent in-feed survival after mechanical mixing in the feed mill. The cryo-protectants showed no significant effects against feed processing, since no difference with or without protectants was observed for both strains (Table 4). The *L. salivarius* strain suffered only from a small numeric decrease in the cell numbers. Similarly, the protectants demonstrated no significant protection effect for the *L. agilis* strain. The refrigerated storage revealed slightly higher viability than storage at room temperature. Short-term storage (day 0–4) showed remarkable in-feed survival rates for both strains without differences of storage with or without cryo-protectants.

As to the mid-term storage (day 5–15), the survival of the *L. salivarius* strain with protectants under a refrigerated condition was higher than without protectants at day 15. However, the difference between the strain with protectants at room temperature and the strain without protectants at a refrigerated condition was not significant. Long-term storage for 28 days showed that the *L. salivarius* strain with protectants at a refrigerated condition exhibited a notably higher survival rate than under any other condition. When incorporated with protectants, the viability of the *L. agilis* strain was significantly higher on day 15 and 28. The details were shown in Table 4.

Table 4. Survival of the probiotic *L. salivarius* and *L. agilis* during the storage [%].

	L. salivarius								***L. agilis***							
	Without Protectants				**With Protectants**				**Without Protectants**				**With Protectants**			
	20 °C		**4 °C**		**20 °C**		**4 °C**		**20 °C**		**4 °C**		**20 °C**		**4 °C**	
BM	9.01 ± 0.04	100.00%	9.00 ± 0.02	100.00%	9.01 ± 0.02	100.00%	9.00 ± 0.04	100.00%	9.02 ± 0.00	100.00%	9.00 ± 0.03	100.00%	9.01 ± 0.03	100.00%	9.00 ± 0.01	100.00%
DPM0	8.97 ± 0.01	91.56%	8.98 ± 0.01	95.33%	9 ± 0.02	97.74%	8.99 ± 0.03	98.00%	9.00 ± 0.00	97.11%	9.00 ± 0.01	100.67%	9.01 ± 0.00	99.02%	9.01 ± 0.01	101.00%
DPM1	8.97 ± 0.04	90.58%	8.98 ± 0.02	95.00%	8.99 ± 0.05	95.48%	8.99 ± 0.03	98.00%	9.00 ± 0.01	96.46%	9.00 ± 0.03	100.33%	9.01 ± 0.01	99.35%	9.01 ± 0.02	101.00%
DPM2	8.96 ± 0.03	89.29%	8.97 ± 0.04	93.33%	8.99 ± 0	95.16%	8.99 ± 0.01	98.67%	8.99 ± 0.04	94.53%	9.00 ±0.04	100.00%	9.00 ± 0.02	97.07%	9.01 ± 0.01	101.00%
DPM3	8.95 ± 0.03	87.34%	8.96 ± 0.04	91.33%	8.98 ± 0.02	93.55%	8.99 ± 0.02	97.00%	8.99 ± 0.02	93.89%	8.99 ± 0.04	98.67%	9.00 ± 0.00	97.07%	9.00 ± 0.03	99.00%
DPM4	8.96 ± 0.02	88.31%	8.96 ± 0.03	91.33%	8.99 ± 0.01	93.55%	8.99 ± 0.01	97.33%	8.99 ± 0.01	94.86%	8.99 ± 0.00	98.00%	8.99 ± 0.03	96.74%	9.00 ± 0.01	99.34%
DPM15	8.91 ± 0.01a	78.90%	8.92 ± 0.03ab	83.67%	8.96 ± 0.02b	89.03%	8.97 ± 0.01b	94.33%	8.95 ± 0.01a	85.21%	8.96 ± 0.02ab	91.00%	8.99 ± 0.01b	95.44%	8.99 ± 0.01b	98.34%
DPM28	8.64 ± 0.05a	42.86%	8.83 ± 0.03b	67.10%	8.84 ± 0.02b	67.74%	8.93 ± 0.01c	85.33%	8.71 ± 0.04a	49.84%	8.85 ± 0.01a	70.33%	8.91 ± 0.02b	79.48%	8.95 ± 0.02b	88.37%

BM = before mixing. DPM = day-post-mixing. a, b, c = significantly different within a row.

3. Discussion

The advantageous role of probiotics in human and animal health has been well accepted. The promising potential is increasingly used in animal nutrition [10]. Among the challenges toward the commercialization of probiotic products, the main factor is the delivery of adequate amounts of viable bacteria at the time of administration [32]. Thus, the optimization of production parameters for specific probiotic strains is of high importance. The current study investigated optimal and cost-effective preparation procedures to ensure a high yield of biomass and maximum in-feed stability of two probiotic strains that were isolated in a previous study. The efficiency of probiotic products is highly dependent on cell viability, since the mode of action of probiotics is conferred by living cells [33]. Thus, a prerequisite for a successful probiotic product is its stability throughout the processing and storage until delivery. Our present study aimed to investigate the optimal and cost-effective preparation procedure for two selected probiotic *Lactobacillus* strains. Aspects of biomass production, protection during lyophilization, and in-feed storage stability were investigated.

The utilization of substrates by lactobacilli is characterized by species-specific or strain-specific differences during growth [34]. To define the specific carbon source preferences of the probiotic *Lactobacillus* strains, the BIOLOG® technology was employed in this study. The microtiter plate-based BIOLOG® methodology is primarily used as a tool for identifying bacteria [35] and has also been used as a tool to compare the metabolic activity of microbial communities from different habitats [36]. The BIOLOG® system is based on the reduction of a redox dye, which indicates bacterial utilization of substrates [37]. Thus, color development during growth not only indicates substrate use, but is also directly proportional to metabolic activity. This potential was used to rapidly identify the preferential substrate utilization of the two probiotic *Lactobacillus* strains. Substrate utilization varied as expected, which shows specific substrate preferences for each strain. After ranking by OD (optical density), the six top substrates were selected for further evaluation.

MRS (de Man, Rogosa, and Sharpe) medium was used in this study, because it is the most commonly used complete medium to allow growth of lactic acid bacteria [38]. The selected carbon sources were added as additional substrates to determine whether they would enhance cell growth on top of the already present glucose. Our results indicate that the addition of sucrose for *L. salivarius* and mannose for *L. agilis* shortened the exponential growth phase and yielded more biomass than with MRS alone.

Lactobacillus spp. are facultative anaerobes, but several species do not tolerate oxygen well [39,40]. Since aerobic cultivation has less energy and is cost intensive, economic advantages can be gained, if technical biomass production can be run under aerobic conditions [41]. Therefore, it was essential to know whether the selected probiotic strains grew equally well under an aerobic condition. As the two strains showed good oxygen resistance, they should be able to be cultured aerobically under large-scale technical conditions. This will lead to a more economic biomass production for those strains.

Extended incubation time (48 h) led to cell loss, which was likely subjected to the self-inhibition caused by accumulation of lactate or other end metabolites [42]. Therefore, biomass production was set to 12 h in the MRS medium supplied with booster substrates. Freeze-drying is one of the commonly employed techniques to produce viable bacterial cells for long-term storage [43,44]. However, a fraction of cells is lost during the lyophilization process because of ice crystal formation with subsequent damage to the viable cell [45]. To maintain viability, a variety of cryo-protectants have been developed to provide structural dry residues as support as well as to act as rehydration receptors [46]. Therefore, cryo-protectants also play an important role in the conservation of probiotic products, which lead to higher survival of probiotic strains [47,48].

Several studies addressed the generation of a protective medium for *L. salivarius* strains, but different methods and optimal media compositions were found in different studies [49–51]. This suggests that protective effects are strain-dependent. To our best knowledge, it is the first study on optimization of cryo-protection for *L. agilis*. Although protection might be strain-dependent, the

beneficial action of skim milk for the *L. agilis* strains may also hold true for other *L. agilis* strains. Thus, future studies on *L. agilis* may also include skim milk as a cryo-protectant during optimization.

Multiple compounds in a cryo-protective mixture were often found to yield synergic effects [52]. Hence, three potential protective factors were used in this study, i.e., skim milk, sucrose, and trehalose. To better understand how the three factors interacted and to find the optimal working concentrations, the Box Behnken Design (BBD) for multivariate optimization schemes with simultaneously changed variables was applied to build a mathematical model with experimental data [27]. The most influencing factor for both strains was skim milk, which is consistent with other investigations [53]. Skim milk for protection of viable cells stabilizes bacterial cell membranes and enables an easier rehydration by creating a high surface porous structure [54]. Both sucrose and trehalose enhanced survival of the cells in addition to the protective effect of skim milk. A similar synergistic effect was reported previously for *Candida sake* cells. In that study, the single use of sucrose did not significantly increase cell viability, but protected the cells better, when skim milk was used during freeze-drying [55].

The protection of bacterial cells by disaccharides is generally attributed to their capacity to hydrate biological structures, which is referred to as a 'water replacement hypothesis' [56]. In studies on the activity of protective sugars, trehalose was shown to be the most effective compound for a range of lactic acid bacterial strains (*L. bulgaricus*, *L. acidophilus*, and *S. salivarius* etc.) [57]. In our case, trehalose did not act as a predominant factor, as demonstrated by a similar effect like sucrose. Between the tested lactobacilli, the *L. salivarius* strain was more dependent on trehalose. Not only the positive influence on viable biomass during the lyophilization, but also improvement of viability during storage has been reported for a range of protectants [58]. Several studies used skim milk, sucrose and trehalose alone or in combination [49,59,60]. To our knowledge, storage in a feed matrix is rarely tested for probiotics in animal nutrition. In one study, a mixture of *Bacillus* spp. was tested as liquid culture in prawn feed. Similar to our study, their results also indicated that probiotic *Bacillus* spp. strains were more stable at a lower temperature (4 °C). Nevertheless, the survival of their isolates at room temperature after 28 days was actually lower than in our study, which can be assigned to a lower stability of liquid cultures compared to dried powders [61].

Storage at 4 °C is not possible for animal feeds, as energy demands for tons of feed would be prohibitively high. Although the temperature exerted a significant impact on survival, it was evident that the combination of protectants enhanced the in-feed stability throughout storage. Furthermore, the *L. salivarius* strain also showed improved stability against physical mixing, when combined with cryo-protectants. On the contrary, the *L. agilis* strain seemed to be more tolerant against a physical force, since no significant changes were observed between cryo-protectants or non-protected feed samples. This corresponds to a report by Sadguruprasad and coworkers (2018) who found highly variable and strain-dependent storage effects on microorganisms [62]. However, the designated protectants in this study benefited the stability of both strains from short-term to mid-term storage when mixed and stored with feed.

4. Materials and Methods

4.1. Strains and Medium

The strains were isolated from broiler intestinal samples and taxonomically identified as *L. salivarius* and *L. agilis* by 16S rDNA sequencing. Both strains were stored in cryo stock at −80 °C. They were cultivated in de Man, Rogosa and Sharpe (MRS, Carl Roth GmbH + Co. KG, Germany) broth in anaerobic jars (Merck KGaA, Germany) with Anaerocult C (Merck KGaA, Germany) at 37 °C for 24 h. The inoculum was prepared fresh each time before use. MRS agar plates were used to determine the viable cell number after treatment.

4.2. Metabolic Fingerprint of Probiotic Lactobacillus Strains

BIOLOG® AN plates (BIOLOG® Inc., Hayward, CA, USA) were used to identify the substrate utilization pattern of the isolates [37]. The technology can also be used to determine substrate utilization patterns of microbial communities [63]. In the present study, the BIOLOG® AN type plate was used to determine the carbohydrate preference of the *Lactobacillus* strains. The procedure followed the manufacturers' guide with a minor modification. Both strains were inoculated in de Man, Rogosa and Sharpe medium (MRS, Carl Roth GmbH + Co. KG, Germany) and incubated in anaerobic jars (Merck KGaA, Germany) with Anaerocult C (Merck KGaA, Germany) overnight. The cultures were then washed with Phosphate Buffered Saline (PBS), pH 7.4, for three times and diluted to 10^7 cells/mL. A total of 100µL bacterial suspension was pipetted into each well of BIOLOG® AN plate in triplicate. The plates were incubated in anaerobic jars with Anaerocult C at 37 °C for 24 h and optical density was read with a microtiter plate reader (Tecan Infinite200Pro, Germany) at OD_{590nm}.

4.3. Booster Effects of Additional Carbohydrate Sources on Biomass Production

Six carbohydrates (sucrose, maltose, mannitol, sorbitol, and melibiose) were selected as possibly beneficial for an increased biomass production of the two probiotic strains. The carbohydrates were added to MRS medium and supplemented with each of the selected additional substrates at a concentration of 1% (*w/v*) and each strain was inoculated into 100 mL of each carbohydrate-supplemented medium reaching a final inoculum of 10^6 CFU/mL. After anaerobic cultivation at 37 °C for 12 h, 24 h, and 48 h, respectively, the resulting biomass was enumerated by plating.

4.4. Determination of Bacterial Growth under Aerobic or Anaerobic Conditions

Pre-cultures of both strains were prepared as described above. An inoculum of each strain was inoculated into 500 mL MRS medium with 10^5 CFU/mL and incubated either in an anaerobic jar with Anaerocult C or in an aerobic incubator at 37 °C. After 12 h of incubation, the biomass of each culture was determined by plating.

4.5. Lyophilization and Optimization of Cryoprotectants

Pre-cultures were harvested after culturing under an aerobic condition at 37 °C for 12 h. Biomass was concentrated by centrifugation (10 min, 15,000 g, 4 °C) and resuspended in different protective media. Each medium contained combinations of sucrose, skim milk, and trehalose at different concentrations (see Supplemental Materials). The suspensions were transferred into lyophilization boxes, incubated at −80 °C for 48 h, and dehydrated at −55 °C in a lyophilizer (LyoVac GT2, LC Didactic, Hürth, Germany) for 48 h. The freeze-dried biomass was ground into powder with a mortar and pestle and stored at 4 °C. The survival of the strain was determined by plating.

The optimization of cryoprotectants was performed using the response surface methodology [64], by which a response surface model was constructed for optimization with a sequential quadratic programming approach.

The survival of both lactobacilli was considered to be an individual response. The Box Behncken Design (BBD) with three factors (skim milk: X_1, sucrose: X_2, and trehalose: X_3) and the software Design Expert 8.06 (Stat-ease Inc., Minneapolis, MN, USA) was used to analyze the survival data. The analytical procedure was referred to a study with minor modification [25]. A three-variable BBD with six replicates at the center point was selected to build the response surface models. The design is shown in the Supplementary Materials (Supplementary Tables S1 and S2). Analysis of variance (ANOVA) was performed to determine the post prediction and reproducibility of assessed combinations. The design was used to determine an optimal composition of protective medium by fitting the polynomial model on the basis of the response surface methodology [65].

4.6. In-Feed Stability of Probiotic Products

Both strains were prepared by lyophilization with or without cryo-protectants, as described above. A basal feed for broiler chicken was produced in mash form in the feed mill of the Institute of Animal Nutrition, Freie Universität Berlin (Supplementary Table S3). The probiotic products were homogenized in the feed with a feed mixer (5 kg) at an approximate concentration of 10^7 CFU/g. The following treatments were applied to the mash feeds: with or without cryo-protectants at room temperature or 4 °C storage. All feed samples were stored for a maximum of 28 days. Subsamples (2 g) were drawn at 0, 1, 2, 3, 4, 15, and 28 days of storage and serially diluted in PBS (Phosphate buffered saline). Residual CFU/g of the strains was determined by plating. The in-feed survival rate was calculated as: survival rate [%] = CFU/g detected at day n post mixing (DPM_n)/CFU/g before mixing (BM) ×100.

4.7. Statistical Analysis

All experiments were performed twice in triplicates. The results are presented as means ± standard deviation (SD). The Design Expert 8.06 software was used for the data analysis estimation of responses and prediction of optimized parameters by plotting response contours and surface graphs. Statistical significances of comparisons were assessed using one-way analysis of variance (ANOVA) or the Mann-Whitney test with the statistics software IBM SPSS (Version 22, SPSS Inc., Chicago, IL, USA).

5. Conclusions

In summary, two broiler-derived probiotic *Lactobacillus* strains (*L. salivarius* and *L. agilis*) were characterized for their preferred substrate utilization, biomass production, and oxygen tolerance as well as their optimal protective agents during freeze-drying and in-feed storage. The response surface methodology was employed to study the optimal composition of protective agents. The prepared probiotic products were supplemented into feed and, although viability decreased, more viable cells were recovered from samples with protectants. This study showed that optimal routines for lab-scale production, processing, and storage of newly-isolated probiotic strains can be employed to increase the technical production of probiotics for poultry nutrition. The results are expected to be further applied for large-scale manufacturing of these probiotic *Lactobacillus* strains.

Supplementary Materials: The following are available online at http://www.mdpi.com/1420-3049/24/18/3286/s1, Figure S1 Linear plot fitting normal plot of residuals. A: *L. salivarius*. B: *L. agilis*. Table S1: Actual and coded levels of variables employed in the Box-Bohnken design. Table S2: Factors and responses of the Box-Behnken design (BBD). Table S3: Composition of feed used for in-feed storage experiments.

Author Contributions: H.R., W.V., and J.Z. organized the whole study. H.R. and W.V. developed the protocol of the method and H.R. performed the experiments and analyzed data. H.R. wrote the first version of the manuscript. W.V. and J.Z. revised the manuscript. All authors read and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Sample Availability: Samples of the compounds are not available from the authors.

CHAPTER 6: Synergistic Effects of Probiotics and Phytobiotics on the Intestinal Microbiota in Young Broiler Chicken

Hao Ren *, Wilfried Vahjen, Temesgen Dadi, Eva-Maria Saliu, Farshad Goodarzi Boroojeni and Jürgen Zentek *

Institute of Animal Nutrition, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany.
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* Correspondence: hao.ren@fu-berlin.de (H.R.); Juergen.Zentek@fu-berlin.de (J.Z.); Tel.: +49-30-83850149 (H.R.); +49-30-38-52256 (J.Z.)

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Hao Ren *, Wilfried Vahjen, Temesgen Dadi, Eva-Maria Saliu, Farshad Goodarzi Boroojeni and Jürgen Zentek *

Institute of Animal Nutrition, Freie Universität Berlin, Königin-Luise-Str. 49, 14195 Berlin, Germany; Wilfried.Vahjen@fu-berlin.de (W.V.); temehi@gmail.com (T.D.); Eva-Maria.Saliu@fu-berlin.de (E.-M.S.); farshad.goodarzi@fu-berlin.de (F.G.B.)

* Correspondence: hao.ren@fu-berlin.de (H.R.); Juergen.Zentek@fu-berlin.de (J.Z.); Tel.: +49-30-83850149 (H.R.); +49-30-38-52256 (J.Z.)

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Abstract: Probiotics and phytobiotics have been studied as in-feed antibiotic alternatives for decades, yet there are no studies on their possible symbiotic effects. In the present study, newly hatched chickens were fed with feeds supplemented either with host-specific *Lactobacillus* strains (*L. agilis* and *L. salivarius*), commercial phytobiotics, or combinations of both. After 13 days of life, crops and caecums were analyzed for bacterial composition (16S rDNA sequencing, qPCR) and activity (bacterial metabolites). Crop and caecum samples were also used to study the ex vivo survival of a broiler-derived extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* strain. In the crop, combinations of probiotics and phytobiotics, but not their single application, increased the dominance of lactobacilli. The single application of phytobiotics reduced the metabolite concentrations in the crop, but certain combinations synergistically upregulated the metabolites. Changes in the qualitative and quantitative composition of the caecal microbiota were less pronounced than in the crop. Acetate concentrations were significantly lower for phytobiotics or the *L. agilis* probiotic strain compared to the control group, but the *L. salivarius* probiotic showed significantly higher acetate concentrations alone or in combination with one phytobiotic. The synergistic effects on the reduction of the ex vivo survival of an ESBL producing *E. coli* strain in crop or caecum contents were also observed for most combinations. This study shows the beneficial synergistic effects of probiotics and phytobiotics on the intestinal bacterial composition and their metabolic activity in young broilers. The reduced survival of potentially problematic bacteria, such as ESBL-producing *E. coli* further indicates that combinations of probiotics and phytobiotics may lead to a more enhanced functionality than their individual supplementation.

Keywords: feed additives; probiotics; phytobiotics; symbiotics; gut microbiota; antibiotic-resistant *Enterobacteriaceae*

1. Introduction

Poultry meat production is expanding rapidly, as global meat consumption is constantly increasing [1]. As antibiotic growth promoters (AGPs) are still used in many countries to increase performance in poultry production, their usage increased simultaneously. However, AGPs contribute to the development and increase of antibiotic-resistant bacteria such as extended-spectrum beta-lactamase (ESBL) producing bacteria in poultry [2]. Many countries have, therefore, banned AGPs for environmental and public health concerns [3,4]. AGPs in poultry production are well known to increase animal performance and infection resistance, and thus, alternatives are demanded to compensate for this loss [5,6]. Among the commercially existing alternatives, probiotics and phytobiotics have been reported to impact on the performance and health in poultry [7].

Regarding probiotics, many publications have shown health promoting effects that are associated with the modifications on gut microbiota [8,9]. *Bacillus* spp., yeasts, and lactic acid bacteria are commonly used as probiotics in animal nutrition. Among the lactic acid bacteria, the lactobacilli have the advantage that they are "generally regarded as safe" (GRAS-status) and are indigenous to the intestinal tract. Certain *Lactobacillus agilis* strains are able to modify the presence of pathogenic bacteria in vitro [10] and ex vivo [11] or regulate the gut microbiota in broiler chickens in vivo [12,13]. In some studies, several *L. salivarius* strains have also been shown to promote animal health [14,15]. Proper supplementation of certain probiotic strains may also lead to the immunomodulation of the host [16], and consequently, resilience against pathogenic bacteria may be increased [17,18]. However, those effects primarily depend on the specific strain, and it is still unclear if immunomodulation is a direct effect of the probiotics or a response to a modified microbiota. Nevertheless, the health-promoting effect of probiotics has often been observed, and this effect may sometimes lead to an improvement in performance. Finally, host-specific probiotics were reported to show better survival and colonization of the strain in the intestinal tract of the host [19,20].

Phytobiotics have also been studied as an alternative to AGPs due to their strong antibacterial activity in vitro and their beneficial influence on animal health and performance in broiler production systems [21–23]. Phytobiotics have the potential to inhibit pathogenic bacteria [24] and show a range of host-related responses such as improvement in antioxidative status [25], intestinal barrier functions [26] as well as a beneficial modification the digestive or immune function of the host [27]. These modes of action may contribute to an increase in growth performance [28,29]. However, like probiotics, due to a lack of studies, it is still unclear if these effects are directly induced by the presence of the phytobiotics or are an indirect effect of changes within the intestinal microbiota.

Synergistic feed additives are thought to act by combining their individual effects that lead to a superior effect than their sole application [30]. This principle has been applied for combinations of probiotics and prebiotics with the intent to promote beneficial bacteria and at the same time, supply suitable substrates for the probiotic [31]. There are also reports on other combinations of feed additives like organic acids combined with phytases [32], probiotics [33], or phytobiotics [34]. To our best knowledge, only one study evaluated the use of a probiotic *E. faecium* strain with a commercial phytobiotic product as a combination in broiler chickens. This study showed beneficial effects on animal health, but no effect on animal performance [35]. In summary, the use of such possibly synergistic combinations of feed additives has not received attention in the field of poultry nutrition. Therefore, a concept was designed and applied in this study to combine host-specific probiotic *Lactobacillus* strains with specific phytobiotics to invoke beneficial synergistic effects for the animal.

Both probiotics and phytobiotics are known to modulate the intestinal microbiota in poultry [36]. Therefore, studies on possible synergistic effects must include an in-depth analysis of the bacterial composition and activity. Furthermore, in light of the evolution of ESBL-producing *Enterobacteriaceae* in poultry [2], the impact of the feed additives on the colonization of antibiotic-resistant bacteria is of high interest.

As young animals are still in the process of developing a mature microbiota [37], this progress may be modified more easily via feed additives [9]. Thus, young broiler chickens were used to investigate the effect of these feed additives on the intestinal microbiota.

Thus, the aim of this study was to compare two different *Lactobacillus* strains and two different commercial phytobiotics as well as their combinations on the possible synbiotic activity in young broiler chickens.

2. Materials and Methods

2.1. Ethics Statement

The feeding trial was conducted according to the German Animal Welfare Act (TierSchG) and approved by the local state office of occupational health and technical safety 'Landesamt für Gesundheit und Soziales, Berlin' (LaGeSo Reg. A 0437/17).

2.2. Animals, Rearing Conditions, and Experimental Diets

Newly hatched Cobb 500 broiler chicks were purchased from a commercial hatchery and randomly allocated into nine experimental groups. All animals were kept in cages and had ad libitum access to feed and water. The ambient temperature was adjusted as follows: for the first 2 days of age, the ambient temperature was 34 °C and was then gradually reduced by 3 °C per week. Artificial light was provided continuously during the first 3 d of age. From 4 d of age onwards, the lighting regime consisted of an 18 h light and 6 h dark cycle. Each group included 21 animals in seven replicate cages per group. Nine experimental feeds were offered in meal form as follows: control feed, two probiotic feeds (10^{10} cfu/kg feed), two phytobiotic feeds (250 mg/ kg feed), and four feeds with the respective combination of probiotics and phytobiotics at the same concentrations. Feed composition and nutrient content of the basal diet are shown in Table 1.

Table 1. Feed ingredients and nutrition composition of the diets (as-fed basis).

Ingredient (%)	
Soybean Meal (49% CP)	32.33
Maize	32.03
Wheat	24.78
Soy oil	5.95
Limestone	1.46
Monocalcium Phosphate	1.84
Vitamin and Mineral Premix	1.20
Salt	0.10
DL-Methionine	0.18
L-Lysine	0.13
Nutrient Composition	
Crude Protein (%)	22.00
Crude Fat (%)	8.19
Crude Fiber (%)	2.42
Methionine (%)	0.51
Lysine (%)	1.28
Threonine (%)	0.84
Calcium (%)	0.96
Phosphorus (%)	0.80
AME_N (MJ/kg)[3]	12.6

1) Contents per kg diet: 4800 IU vit. A; 480 IU vit. D3; 96 mg vit. E (α-tocopherole acetate); 3.6 mg vit. K3; 3 mg vit. B13 mg vit. B2; 30 mg nicotinic acid; 4.8 mg vit. B6; 24 µg vit. B12; 300 µg biotin; 12 mg calcium pantothenic acid; 1.2 mg folic acid; 960 mg choline chloride; 60 mg Zn (zinc oxide); 24 mg Fe (iron carbonate); 72 mg Mn (manganese oxide); 14.4 mg Cu (copper sulfate-pentahydrate); 0.54 mg I (calcium Iodate; 0.36 mg Co (cobalt- (II)-sulfate-heptahydrate); 0.42 mg Se (sodium selenite); 1.56 g Na (sodium chloride); 0.66 g Mg (magnesium oxide). 2) Nitrogen-corrected apparent metabolizable energy estimated from the chemical composition of the feed ingredients (based on the European Union (EU) Regulation - Directive 86/174/EEC): 0.1551 × % crude protein + 0.3431 × % ether extract + 0.1669 × % starch + 0.1301 × % total sugar.

2.3. Sampling

On the 13th day of life, two birds from each group were randomly selected daily, weighed, and sacrificed by exsanguination after anesthesia. In order to obtain fresh digesta samples for ex vivo studies and their appropriate processing, this procedure was continued until 10 animals per group were

sampled in a span of five days. The digesta from crop and caecum of birds were collected individually and allocated into two portions. One portion was snap-frozen in liquid nitrogen for DNA extraction and metabolite analysis; the other portion was used for ex vivo studies with an ESBL-producing *E. coli* strain.

2.4. Bacteria and Media

Probiotic LS1 (*L. salivarius*) and LA73 (*L. agilis*) were isolated, identified, and characterized previously [38]. Both strains were cultured anaerobically (starting culture) or aerobically (biomass production) in de Man, Rogosa, and Sharpe broth (MRS, Carl Roth GmbH + Co. KG, Germany) at 37 °C for 24 h.

The ESBL-producing *E. coli* (EE10716) strain was isolated from the broiler chicken by the Institute of Microbiology and Epizootics of the Freie Universität Berlin within the RESET project (Germany, http://www.reset-verbund.de/index.htm) and produced the CTX-M-15 lactamase [39]. This strain was selected as the model strain to study its survival in ex vivo broiler ingesta samples. The strain was stored as cryo stock and cultured in brain heart infusion broth (BHI, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for further application.

2.5. Feed Additives

The probiotic *Lactobacillus* cells were harvested from MRS broth after aerobic growth at 37 °C for 12 h. The pelleted biomass was concentrated by centrifugation (10 min, 15,000× *g*, 4 °C) and freeze-dried at –55 °C in a lyophilizer (LyoVac GT2, Hürth, Germany) for 48 h after pre-freezing at –80 °C as described previously [40]. The lyophilized probiotics were stored at 4 °C until mixed with the feed.

The commercial phytogenic compounds (formulation C and L) were provided in solid form by EW nutrition (Visbek, Niedersachsen, Germany) and kept at 4 °C until mixed with feed. The active ingredients in formulation C were carvacrol and cinnamaldehyde, while formulation L contained carvacrol, cinnamaldehyde, and eugenol. All additives were mixed with the basal diet using a feed mixer and stored at room temperature. To ensure that sufficient amounts of viable probiotic cells and concentrations of the volatile phytobiotic products were present in the feeds, the diets were prepared on a weekly basis.

2.6. 16S rDNA Sequencing and qPCR

Total DNA was extracted from 0.2 g digesta samples of crop and caecum (45 samples for each section, five samples for each group) with a commercial extraction kit (QIAamp Fast DNA stool mini kit, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions with minor modification (lysis step at 90 °C). The resulting DNA extracts were stored at –30 °C until further analysis.

DNA extracts were subjected to amplicon sequencing using an Illumina NextSeq500 sequencer (LGC, Berlin, Germany) with two 150–base pair reads. After a combination of forward and reverse reads using the BBMerge tool [41] and demultiplexing, the resulting 16S-rDNA sequences were analyzed using the QIIME2 pipeline [42] and the SILVA SSU database [43]. Quality control and determination of sequence counts were performed using the DADA2 [44]. Sequence variants with less than five counts were excluded from further analysis to increase the confidence of sequence reads and reduce bias by possible sequencing errors [45]. Normalization of sequence reads was done by verification with an equal representation of 10,000 sequences per sample [46]. Further statistical analysis was done using RStudio (Rstudio, Boston, USA) and KNIME 4.01 (KNIME, Zürich, Switzerland) [47] with the R package stats and ggplot2.

Quantification of predominant bacterial groups and species in poultry was performed via qPCR assays with five biological replicates per group (Table S1). Target gene copy numbers were calculated from standard curves with known copy number concentrations.

2.7. Analysis of Bacterial Metabolites

Short-chain fatty acids (SCFA) in crop and caecum contents were analyzed as described previously [48]. In short, SCFAs were analyzed using gas chromatography (Agilent Technologies 6890N, autosampler G2614A, and injection tower G2613A; Network GC Systems, Böblingen, Germany) equipped with a flame ionization detector. D- and L-lactate was measured by HPLC (Agilent 1100; Agilent Technologies, Böblingen, Germany) with a pre-column (Phenomenex C18 4.0 4.0 × 2.0 mm; Phenomenex Ltd., Aschaffenburg, Germany) and an analytical column (Phenomenex Chirex 3126 (D)-penicillamine 150 × 4.6 mm; Phenomenex Ltd.).

2.8. Ex-vivo Survival of the ESBL-Producing E. coli Model Strain in Crop and Caecum

The ex vivo survival of the ESBL-producing *E. coli* model strain was evaluated according to a previous study with minor modification [49] in a two-step incubation assay. In short: in the first step, fresh crop and caecum samples were immediately diluted anaerobically in incubation buffer at 1:5 (*w/v*). After 5 min sedimentation, supernatants (190 µL) were anaerobically transferred to microtiter plates, and the *E. coli* strain EE10716 was added (a final concentration of 2×10^5 cells/mL). To avoid the interference from the indigenous ESBL-producing bacteria in the samples, identical plates but without inoculation of the ESBL indicator strain was prepared and incubated under the same conditions. In the second step, after anaerobic incubation at 37 °C for 12 h, samples were transferred to a microtiter plate containing Mueller–Hinton broth 2 (Carl Roth GmbH + Co. KG, Germany) with 4 µg/ml cefotaxime to ensure selective growth of the resistant *E. coli* strain. The plates were then incubated aerobically in a microtiter plate reader (Tecan infinite M Plex, Männerdorf, Switzerland) at 37 °C and OD_{690nm} was obtained as the $OD_{experiment}$ subtracted by the $OD_{control}$. The resulting growth curves were subjected to a non-linear regression model using the Gompertz equation, which gave the best fit with an $r^2 \geq 0.98$ for all samples. The lag time was then documented as an indicator for *E. coli* growth inhibition. All assays were performed with five technical replicates.

2.9. Statistical Analysis

Results are presented as means and pooled standard error of mean except for figure 2, which is presented as the means and standard deviation. Due to the non-normally distributed nature of the data, we chose to use the Kruskal–Wallis test, followed by the Mann–Whitney test, when appropriate. The Chi-square test was performed to compare *Clostridium. perfringens* (*C. perfringens*) counts. Statistical procedures were performed at a significance level of 95% using the SPSS Statistics software (SPSS, Chicago, USA).

3. Results

3.1. Qualitative Determination of the Intestinal Microbiota in Young Broiler Chickens

A total of 1.26×10^6 quality sequence reads from 89 samples (44 crop samples and 45 ceca samples) with a mean combined read length of 404 nucleotides were used for the qualitative analysis of the bacterial composition. One sample (control group, crop) had to be omitted from the analysis, as it contained a massive amount of *Aeromonas* spp., and *C. perfringens*, which classified this sample as a true outlier.

A total of six phyla, 18 orders, and 88 genera were assigned to the sequences. A comprehensive overview of the taxonomic assignments is given in Tables S2–S7.

Overall, the crop was heavily dominated by *Lactobacillus* spp., while caecal contents displayed a more even distribution with an unidentified *Clostridiales*, *Bacteroides* spp., and *Faecalibacterium* spp. as the most dominant genera (Figure 1).

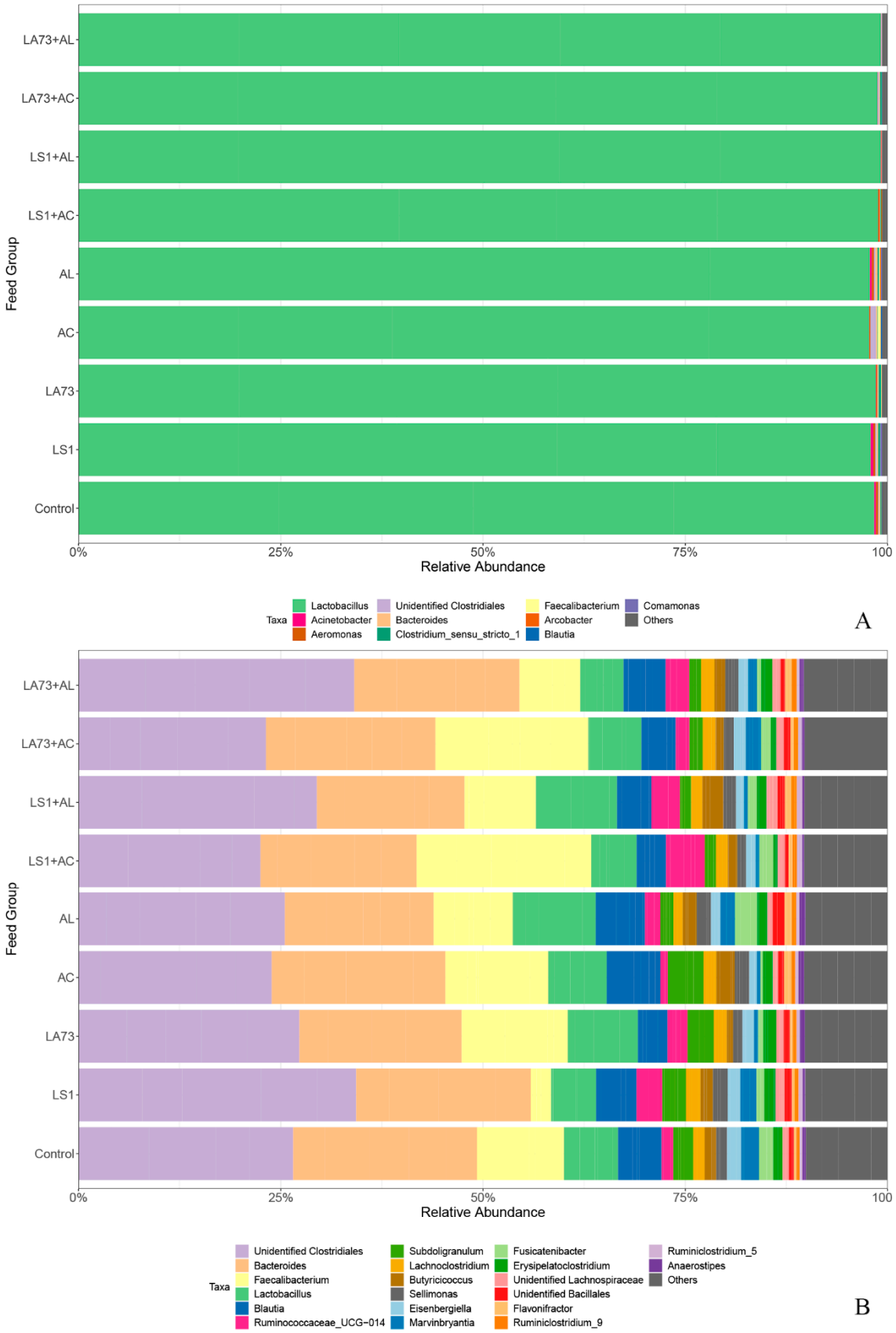

Figure 1. Phylogenetic composition of the intestinal microbiota in young broiler chickens. (**A**) Genus distribution in the crop; (**B**) Genus distribution in the caecum (LS1: *L.salivarius*, LA73: *L. agilis*, AC: formulation C, AL: formulation L). Data of each group are presented as the mean of five samples.

3.2. Impact of Probiotics and Phytobiotics on the Relative Composition of the Crop Microbiota

The crop was considerably dominated by *Lactobacillus* spp. sequences in all feeding groups, ranging from 98.0% to 99.7% abundance (Table S6). Other dominating genera, including *Aeromonas* spp., *Acinetobacter* spp., *Bacteroides* spp., as well as two unidentified genera belonging to the *Clostridiales* order rarely exceeded one percent of all sequences. The control group, as well as treatment groups with single feed additives, showed the lowest lactobacilli abundance. However, combinations of probiotics and phytobiotic additives significantly increased lactobacilli abundance at the expense of the next dominant genera (*Aeromonas*, *Acinetobacter, Bacteroides*) compared to control. One exception was the combination of LA73 and formulation C, in which the *Clostridium sensu stricto 1* genus and *Bacteroides* spp. prevailed. Seven different *Lactobacillus* species were assigned based on their unique OTUs, which significantly differed in their abundance in the crop (Table 2). As expected, supplementation of the probiotic *Lactobacillus* strains significantly increased their abundance in the respective treatment groups. Another dominating *Lactobacillus* species (*L. crispatus*) showed a significant numerical reduction in all treatment groups compared to the control.

Interestingly, the putative pathogenic genera *Aeromonas* spp. and *Acinetobacter* spp. were among the dominating sequences in the crop of 13-day-old animals. No significant differences were observed in this genus, but combinations of probiotics and phytobiotic additives strongly reduced the numerical abundance of *Aeromonas* spp., except for the combination of LS1 and formulation C. The genus *Acinetobacter* spp. was strongly reduced in the LA73 and formulation C groups, as well as in all combination groups (see Table S6).

Ecological indices showed no significant differences for richness (number of different sequences), but diversity (Shannon Index) and accordingly evenness were significantly reduced in treatment groups supplemented with the phytobiotic formulation C as well as the combination of the probiotic LS1 and formulation L (Table S8).

Table 2. Relative abundance of dominant putative *Lactobacillus* species in the crop of 13-day-old broiler chickens fed probiotics and phytobiotics [%].

Putative species name	Control	LS1	LA73	Formulation C	Formulation L	LS1 & Formulation C	LS1 & Formulation L	LA73 & Formulation C	LA73 & Formulation L	Pooled SEM	*p*-value[1]
L. salivarius	33.8^{a,b}	46.5^{a,b}	24.9^{a,b}	68.2^{b,c}	26.3^{a,b}	57.1^{b,c}	71.9^{c}	18.1^{a}	37.6^{b}	3.21	0.002
L. agilis	16.1^{a,b}	9.1^{a}	38.1^{b}	4.5^{a}	38.3^{b}	13.3^{a,b}	5.7^{a}	49.4^{c}	20.8^{a,b}	0.97	0.003
L. crispatus	18.2^{c}	10.3^{b,c}	9.9^{b,c}	8.7^{b,c}	5.7^{a}	7.1^{a}	3.2^{a}	13.8^{b,c}	8.4^{b}	0.68	0.049
L. reuteri	15.3	18.0	15.2	6.2	14.0	10.9	8.2	7.2	16.5	0.61	0.425
L. johnsonii	8.7	9.8	8.0	9.5	11.6	9.8	8.7	5.2	11.1	1.39	0.593
L. kitasatonis	4.4	4.3	1.1	0.6	1.9	0.4	1.2	n.d.	0.7	3.73	0.814
L. vaginalis	3.4	1.9	2.9	2.4	2.1	1.5	1.1	6.3	4.7	0.43	0.120

n.d. = Not detected; [1] = Kruskal–Wallis Test; [a,b] = different superscripts denote significant changes within a row (Mann–Whitney U test, $p \leq 0.05$).

3.3. Impact of Probiotics and Phytobiotics on the Relative Composition of the Caecal Microbiota

An unidentified *Clostridiales* genus and *Bacteroides* spp. were the most dominant genera in the caecum, followed by *Faecalibacterium* spp. (Table S7). Due to high individual differences in the samples, only a few significant differences were observed. There was a trend for an increased abundance of the unidentified *Clostridiales* genus in the probiotic LS1 and in the LA73/ formulation L treatment groups, which was offset by a significant reduction of *Faecalibacterium* spp. in these groups. Additionally, compared to control- or phytobiotic supplemented feed groups, *Anaerostipes* spp. showed significant differences in feed groups that were supplemented with the probiotic strain LS1 alone or in combination.

Ecological indices did not differ significantly between treatment groups (Table S9). A range of 40.6 to 45.8 independent OTU were found with even distributions of diversity and very similar evenness.

3.4. Quantitative Determination of the Intestinal Microbiota in Young Broiler Chickens

16S rDNA sequencing yields an in-depth view of the abundance of bacterial genera but is unable to quantify the bacterial composition in the intestinal tract. Therefore, qPCR assays on a range of dominant bacterial groups and species were carried out.

3.5. Impact of Probiotics and Phytobiotics on Dominant Bacterial Groups and Species in the Crop

The quantitative determination of the crop microbiota confirmed the dominance of lactobacilli and mirrored results for relative *Lactobacillus* spp. abundance (Table 3). The most prominent species was *L. salivarius*, followed by *L. reuteri*, *L. agilis* and *L. acidophilus*. As expected, supplementation of LS1 significantly increased the *L. salivarius* counts, while LA73 significantly increased *L. agilis* copy numbers. This was also visible for their combinations with the phytobiotic additives. Interestingly, the supplementation of the probiotic *L. salivarius* strain reduced the counts of the *L. agilis* species and vice versa. The other dominant lactobacilli (*L. reuteri* and *L. acidophilus*) showed no significant response to the presence of either probiotic supplementation.

Table 3. Quantitative determination of the crop microbiota of 13-day-old broiler chickens fed different probiotics and phytobiotics [log 16S rDNA copy number/g].

	Control	**LS1**	**LA73**	**Formulation C**	**Formulation L**	**LS1 & Formulation C**	**LS1 & Formulation L**	**LA73 & Formulation C**	**LA73 & Formulation L**	**Pooled SEM**	***p*- value[1]**
Lactobacillus spp.	9.84	9.81	10.07	9.81	9.77	9.81	10.01	9.89	10.00	0.04	0.167
L. salivarius	9.14^{a}	9.55^{b}	$9.39^{a.b}$	$9.58^{b.c}$	9.25^{a}	$9.6^{b.c}$	9.92^{c}	9.20^{a}	$9.53^{a.b}$	0.05	0.016
L. agilis	8.87^{b}	8.83^{b}	9.30^{d}	8.19^{a}	9.14^{c}	8.73^{b}	8.61^{b}	9.40^{d}	9.13^{c}	0.11	0.016
L. reuteri	9.19	9.23	9.61	8.91	9.08	9.11	9.23	9.29	9.52	0.07	0.338
L. acidophilus	8.45	8.33	8.58	7.99	8.12	7.98	8.08	7.91	8.10	0.11	0.768
Clostridial Cluster XIVa	8.38^{b}	8.41^{b}	8.45^{b}	8.60^{b}	8.43^{b}	7.76^{a}	7.88^{a}	8.49^{b}	8.22^{b}	0.09	0.040
Clostridial Cluster IV	8.08	7.89	8.11	8.16	7.86	7.36	7.43	7.92	7.72	0.09	0.206
Clostridial Cluster I	7.41^{b}	7.32^{b}	7.81^{c}	$6.95^{a.b}$	7.27^{b}	6.99^{b}	7.27^{b}	7.03^{b}	6.62^{a}	0.08	0.016
BPP-Cluster[2]	8.48	8.36	8.56	8.22	8.37	7.95	8.16	8.29	7.82	0.07	0.338
Enterobacteria[3]	$8.83^{a.b}$	8.42^{a}	8.94^{b}	8.64^{a}	8.38^{a}	8.30^{a}	8.47^{a}	$8.62^{a.b}$	8.45^{a}	0.04	0.004
Escherichia group	6.89^{a}	$6.66^{a.b}$	7.28^{c}	$6.93^{a.b}$	6.27^{a}	6.06^{a}	6.62^{a}	$7.01^{b.c}$	6.40^{a}	0.08	0.018
Int1[4]	7.77^{b}	7.37^{b}	7.98^{c}	7.27^{b}	7.34^{b}	7.17^{b}	7.27^{b}	$6.99^{a.b}$	6.72^{a}	0.08	0.042

[1] = Kruskal–Wallis Test; [2] = *Bacteroides-Prevotella-Porphyromonas* Cluster; [3] = Copy number of the enterobacterial ribosomal polymerase beta subunit; [4] = Enterobacterial Integrase 1 gene.

The combination of LS1 with both formulation C and formulation L significantly reduced bacteria belonging to the Clostridial Cluster XIVa, while no changes were observed in any other feeding group. The copy numbers of the total enterobacteria and the *Escherichia* group were higher in the LA73 group, but absolute differences were only minor. However, in combination with either phytobiotic additive, a small (formulation C) or drastic (formulation L) reduction was observed for *Escherichia* group counts. Similarly, the copy numbers of the integrase 1 gene, responsible for the incorporation of foreign DNA, mirrored the trend seen for the *Escherichia* group.

Finally, due to the presence of an unidentified *Clostridium sensu stricto* 1 in the sequencing data, we also tested for the presence of *C. perfringens*, which is phylogenetically closely related to this genus (Table S10). *C. perfringens* was detected sporadically in the crop samples, but no significant differences were observed ($p = 0.103$, Chi-square test). However, all the samples from the feed groups supplemented with LA73 or its combination with formulation L were negative for *C. perfringens*. Interestingly, the omitted sample from the control group showed a very high amount of *C. perfringens* (log 6.1).

3.6. Impact of Probiotics and Phytobiotics on Dominant Bacterial Groups and Species in the Caecum

The supplementation of probiotics and phytobiotics showed no significant impact on the caecal microbiota (Table 4). Two clostridial clusters, the *Bacteroides-Prevotella-Porphyromonas* cluster, and enterobacteria followed by lactobacilli dominated the caecum of 13-d old broiler chickens. *C. perfringens* was detected in all control samples (see Table S10), but only rarely in the other experimental groups. However, no significant differences between the feed groups were observed ($p = 0.126$, Chi-square test).

Table 4. Quantitative determination of the caecal microbiota of 13-day-old broiler chickens fed different probiotics and phytobiotics [log 16S rDNA copy number/g].

	Control	**LS1**	**LA73**	**Formulation C**	**Formulation L**	**LS1 & Formulation C**	**LS1 & Formulation L**	**LA73 & Formulation C**	**LA73 & Formulation L**	**Pooled SEM**	***p*- Value[1]**
Clostridial Cluster XIVa	10.96	10.97	10.85	11.15	11.09	11.08	11.17	10.93	11.11	0.05	0.639
Clostridial Cluster IV	10.39	10.28	10.51	10.72	10.61	10.73	10.67	10.47	10.30	0.05	0.134
Clostridial Cluster I	6.45	6.13	5.93	6.33	6.15	6.76	6.70	5.60	6.03	0.14	0.383
BPP-Cluster[2]	10.72	10.77	10.72	10.86	10.72	10.72	10.86	10.66	10.69	0.03	0.650
Lactobacillus spp.	9.63	9.61	9.82	9.73	9.83	9.69	9.94	9.61	9.67	0.04	0.605
Enterobacteria[3]	10.51	10.49	10.43	10.43	10.36	10.35	10.75	10.30	10.53	0.06	0.857
Escherichia group	8.45	8.43	8.32	8.21	8.53	8.13	8.13	8.20	8.43	0.07	0.910
L. salivarius	8.78	8.94	8.95	9.20	9.14	9.17	9.32	8.70	8.94	0.05	0.130
L. agilis	9.42	9.17	9.54	9.44	9.47	9.50	9.75	9.21	9.51	0.07	0.392
L. reuteri	8.79	8.71	9.10	8.27	8.86	8.18	8.12	9.14	9.05	0.07	0.774
L. acidophilus	8.26	7.48	8.21	8.12	8.49	8.27	8.27	7.67	7.79	0.11	0.620
Int1[4]	7.94	7.69	7.35	7.76	7.75	7.80	7.27	7.58	7.95	0.11	0.177

[1] = Kruskal–Wallis Test; [2] = *Bacteroides-Prevotella-Porphyromonas* Cluster; [3] = Copy number of the enterobacterial ribosomal polymerase beta subunit.

3.7. Bacterial Metabolism of the Intestinal Microbiota in Young Broiler Chickens

Short-chain fatty acids and lactate concentration in the crop are shown in Table 5. As expected, the crop was dominated by lactate in all feeding groups, while acetate and propionate only played minor roles in the formation of bacterial metabolites. The phytobiotic supplementation significantly reduced lactate concentrations in the crop, and also showed the numerically lowest concentrations of acetate. Surprisingly, the single supplementation of the probiotic lactobacilli did not increase lactate concentrations compared to the control. However, the combination of LA73 and formulation L led to significantly more lactate than in any other treatment group. The combination of the probiotic LS1 with phytobiotic products also led to higher lactate concentrations than their single addition.

Lactate in the caecum is not shown, as those values are generally very low or undetectable. The dominating SCFA in the caecum was acetate (Table 6). The phytobiotic products again showed significantly (formulation C) or numerically but non-significantly (formulation L) lower acetate concentrations compared to the control group. The addition of the probiotic strain led to a diverse bacterial response in the caecum, as the strain LS1 showed a significantly increased acetate concentration, while L73 led to significantly lower acetate concentrations compared to the control group. However, all combination groups displayed significantly or numerically higher concentrations than their respective single addition except for the combination of LS1 and formulation C.

Table 5. Concentration of lactate and short-chain fatty acid (SCFA) in the crop of 13-day-old broiler chickens fed different probiotics and phytobiotics [µmol/g].

	Control	LS1	LA73	Formulation C	Formulation L	LS1 & Formulation C	LS1 & Formulation L	LA73 & Formulation C	LA73 & Formulation L	Pooled SEM	*p*-Value[1]
L-lactate	19.8^{b}	15.2^{a,b}	21.1^{b}	11.2^{a}	14.2^{a}	19.5^{b}	25.0^{b}	16.9^{b}	29.5^{c}	1.10	0.008
D-lactate	11.5^{b}	3.9^{a}	6.6^{a,b,c}	2.2^{a}	4.2 ^{a}	4.9^{a,b}	7.6^{b}	4.8^{a}	13.2^{c}	0.70	0.029
total Lactate	31.3^{b,c}	19.1^{b}	27.8^{b,c}	13.4^{a}	18.3^{a}	24.4^{a,b}	32.6^{b}	21.7^{b}	42.7^{c}	1.69	0.010
Acetate	5.9	4.4	6.0	2.4	3.3	4.2	4.1	4.1	6.7	0.49	0.638
Propionate	1.5	1.8	2.1	2.0	1.9	1.9	1.9	1.7	1.7	0.04	0.218
n-butyrate	0.1	n.d.[2]	0.1	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	0.19	0.332
i-valerate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		0.317
n-valerate	n.d.	n.d.	0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		0.277
Total SCFA	7.5	6.3	8.2	4.3	6.1	6.1	6.0	5.8	8.4	0.51	0.719
Total Metabolites[3]	40.4^{b}	25.4^{a,b}	35.9^{b}	18.9^{a}	24.5^{a,b}	33.1^{b}	39.5^{b}	29.0^{a,b}	53.7^{c}	2.20	0.020

[1] = Kruskal–Wallis Test; superscripts denote significant differences within a row (Mann–Whitney U Test, $p \leq 0.05$); [2] = not detected; [3] = sum of the total lactate and total SCFA.

Table 6. Concentration of SCFA in the caecum of 13-day-old broiler chickens fed different probiotics and phytobiotics [µmol/g].

	Control	LS1	LA73	Formulation C	Formulation L	LS1 & Formulation C	LS1 & Formulation L	LA73 & Formulation C	LA73 & Formulation L	Pooled SEM	*p*-Value[1]
Acetate	39.9^{b}	55.8^{c}	32.1^{a}	30.7^{a}	34.8^{a,b}	53.1^{c}	43.6^{b}	42.6^{b}	44.0^{b}	2.01	0.044
Propionate	5.0	7.8	6.1	5.2	4.2	5.7	5.2	5.9	7.0	0.28	0.142
i-butyrate	1.9	2.1	0.8	0.6	1.2	1.1	0.4	0.5	0.6	0.22	0.963
n-butyrate	8.7	7.7	5.8	7.7	6.0	9.4	8.7	10.1	6.2	0.50	0.548
i-valerate	0.5	0.3	0.3	0.4	0.2	0.4	0.2	0.3	0.2	0.03	0.684
n-valerate	0.4	0.4	0.6	0.6	0.3	0.4	0.3	0.2	0.3	0.04	0.504
BCFA[2]	2.0	2.4	1.1	0.8	1.2	1.5	0.6	0.6	0.7	0.21	0.873
Total SCFA	55.9	74.1	45.6	44.9	46.4	70.1	58.4	59.3	58.2	2.50	0.103

[1] = Kruskal–Wallis Test; superscripts denote significant differences within a row (Mann–Whitney U Test, $p \leq 0.05$).; [2] = Sum of branched chain fatty acids.

3.8. Ex-vivo Growth Response of an ESBL Producing E. coli Model Strain in Intestinal Samples

The impact of different intestinal conditions due to the addition of probiotics and phytobiotics was tested for the ex vivo survival of an ESBL-producing *E. coli*. Lag time, i.e., start of the exponential growth phase, is the most informative fitness parameter of a bacterium, and thus, this parameter was used to estimate the impact of probiotics and phytobiotic supplementation.

In crop samples, the significantly lowest lag time, and thus, the best fitness of the *E. coli* strain was observed in the LS1 treatment group, followed by the formulation L (Figure 2A). However, the combination of both additives slightly increased lag time compared to a single application. The significantly lowest fitness was noted in the combination of LA73 and formulation L, although their single application yielded a significantly higher fitness.

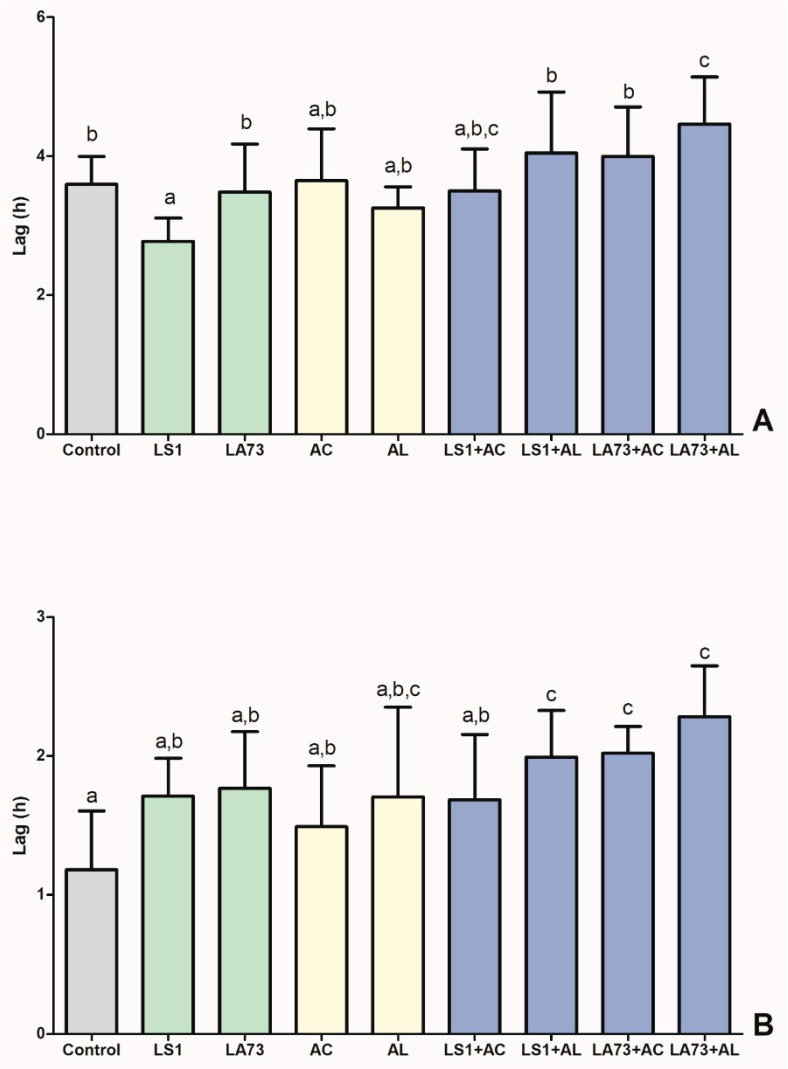

Figure 2. Ex vivo survival of an ESBL-producing *E. coli* strain in crop- or ceca contents of 13-day-old broiler chickens. (**A**) crop; (**B**) caecum (LS1: *L.salivarius*, LA73: *L. agilis*, AC: formulation C, AL: formulation L). Grey: Control, Green: Probiotic supplementation, Yellow: Phytobiotic supplementation, Blue: Combined feed additive supplementation. Data are presented as means with standard deviation.

In caecal samples, the best survival of the *E. coli* strain was observed in the control group (Figure 2B). Both additive types showed a numerically lower fitness as single supplementation, but combinations of probiotics and phytobiotics displayed a significantly lower survival with the exception of the combination of LS1 and formulation C.

4. Discussion

Accumulating numbers of studies show that novel additives such as probiotics or phytobiotics may be used as alternatives to in-feed AGPs. However, the efficiency of the alternative additives depends on many factors like uptake concentration, overall diet, supplementation method, or the rearing environment [50]. To maximize the efficiency of those alternatives, the combination in accordance with a synergistic concept is a favorable solution that may act beyond their single applications. The present study investigated the synergistic effects of probiotics and phytobiotic feed additives on the intestinal microbiota in young broiler chickens. The gut microbiome is a key to understand animal health and nutrition better [51], and thus, this study focused on the bacterial composition and –activity in crop and caecum of young broiler chickens that has not yet developed a stable microbiota.

Probiotics generally do not reduce the total amount or activity of bacteria in the gut, but can sometimes increase bacterial metabolite concentrations in broiler chicken [52,53]. On the other hand, phytobiotics are often used due to their strength in vitro antibacterial activity [54]. The active ingredients in the phytobiotic products were carvacrol and cinnamaldehyde as well as additionally eugenol in formulation L. All three substances have been shown to inhibit a range of bacteria in vitro and show diverse effects on performance, immunology, and reduction of pathogenic bacteria in broiler chicken [55–57]. The probiotic *Lactobacillus* strains in this study were previously characterized in vitro regarding their resilience against both phytobiotic products (data not shown). Both strains showed a high tolerance in vitro, which could be confirmed in vivo, especially for the *L. salivarius* strain. In fact, a strong synergistic effect was observed for the species *L. salivarius* with formulation L, which may indicate that the functionality of LS1 increased accordingly when applied as a synergistic product. However, the species *L. agilis* was strongly inhibited by the effects of the formulation C in vivo, but in combination with LA73 an increase of this species was observed in the crop. This also points to a synergistic effect for increased LA73 colonization in combination with formulation C. Taken together; the data suggest that indigenous *L. agilis* strains may be much more sensitive to phytobiotic pressure compared to the supplemented *L. agilis* strain. This reflects strain-specific differences in lactobacilli in general. Therefore, synergistic effects seem to be in effect regarding certain combinations of probiotic and phytobiotics. Unfortunately, to the best knowledge of the authors, there are not many reports on phytobiotic modifications of the intestinal microbiota in broiler chicken, studies on combined usage with probiotics are even rare [35]. However, studies in humans, pigs, and rats show that the absorption of the mentioned essential oils occurs in the upper small intestine [58,59]. It is therefore probable that pancreatic enzymes in poultry attack these substances, and resorption of their metabolites could be expected before they reach the caecum. Consequently, it is unlikely that relevant concentrations of the phytobiotics reached the hindgut. This implies that results on crop and caecum microbiota should be viewed separately from different angles and that modifications of the caecal microbiota are largely due to bacterial- or host-related changes in the upper intestinal tract.

The crop plays an essential role in the transient storage and moisturization of feed [60]. It is also viewed as a pre-gastric fermentation chamber that defines the input of bacteria into the gut [61]. Generally, the crop of broiler chicken is heavily dominated by certain dominant *Lactobacillus* species [62–64], which was also observed in this study. Of the few studies on the subject, one report with a probiotic *L. salivarius* strain showed no effect on crop lactobacilli after administration [65]. This was also observed for the number of lactobacilli in this study, but the single addition of probiotic strains significantly enhanced their quantity compared to the control group. Thus, both strains were able to colonize the crop. This was not unexpected, as both probiotic strains were originally isolated from the broiler intestine and already demonstrated great potential for in vitro survival under-stimulated gastric stress and epithelial adherence in our previous study [38].

Significant positive synergistic effects on relative *Lactobacillus* spp. abundance were only observed for *L. salivarius* in combination with formulation L as well as for *L. agilis* with both phytobiotics. In general, the supplementation of the probiotic strains seemed to be the overriding effect on *Lactobacillus* spp. abundance, while the additional phytobiotic supplementation showed only minor effects. Similarly,

a slight non-significant decrease of species richness was observed for combination groups, but significant differences for microbiota diversity (Shannon-index) did not show clear synergistic effects.

The impact of the probiotics and phytobiotics on the crop microbiota also extended to non-dominant bacteria. For instance, the *Clostridium sensu stricto* 1 genus exhibited the highest abundance apart from the lactobacilli. This *Clostridium* genus has been shown to be associated with necrotic enteritis and *Clostridium perfringens* infection models [66,67]. However, the *Clostridium sensu stricto* 1 cluster also contains species such as *C. butyricum*, which has also been used as a probiotic in poultry [68]. It is, therefore, difficult to assign a positive, indifferent or negative role to this genus. Nevertheless, the comparison of *Clostridium sensu stricto* 1 sequencing data to the much more sensitive *C. perfringens* qPCR data did not show any correlation (data not shown). We can, therefore, conclude that this genus probably did not include *C. perfringens*. The abundance of *Clostridium sensu stricto* 1 was high in single probiotic and formulation L supplemented feed groups but was dramatically reduced in combinations of LS1 with both phytobiotic products and especially in LA73 with formulation L. Thus, synergistic effects in the significant reduction of this *Clostridium* spp. were visible only for certain combinations. Although the additional eugenol in formulation L may have played a role in enhancing the *Clostridium sensu stricto* 1 abundance compared to formulation C in single supplementation; this does not account for its total inhibition in combination with both probiotic strains. These results signify again that the synergistic mode of action on certain bacteria are not additive but rely on the impact of the feed additives on other bacteria. In this case, the concomitant responses of *Faecalibacterium* spp., *Blautia* spp., and an unidentified *Clostridiales* may have played a role in the significant modification of *Clostridium sensu stricto* 1.

Interestingly, similar changes in relative abundance were observed for the putatively pathogenic genera *Aeromonas* spp. and *Acinetobacter* spp. Furthermore, *C. perfringens* positive samples in the crop were generally lower in combination groups, which points to their potential to a synergistic potential to reduce detrimental bacteria in the intestinal tract. Nevertheless, synergistic effects for these bacteria were not visible for all combinations. Therefore, the response of the intestinal microbiota to different phytobiotics seems to be quite diverse. However, as beneficial synergistic effects are clearly visible regarding putatively pathogenic bacteria, the combination of certain probiotic and phytobiotic products may be advantageous for animal health.

Overall, bacterial activity in terms of bacterial metabolites was lower in the crop of feed groups with single phytobiotic addition, although only the reduction in lactate was significant. Carvacrol, cinnamaldehyde, and eugenol are all known to inhibit bacterial growth in vitro, and consequently, their activity [69,70]. Our results indicate that both phytobiotic formulations indeed inhibited bacterial metabolism, although no significant changes in the absolute bacterial counts were observed. Consequently, at the employed in-feed concentrations, the phytogenic products may not inhibit total bacterial growth *per se*, but significantly reduce their activity in the crop. However, the production of lactate or acetate in the intestinal tract is usually considered beneficial, as it may inhibit pathogenic or other bacteria detrimental to the host [71,72]. The increased lactate concentration in certain combinations of phyto- and probiotics points to a beneficial synergistic effect. Still, the synergism seems to depend on specific combinations and cannot be classified as an additive effect of individual supplementation.

We also monitored the ex vivo survival of an ESBL producing, but non-pathogenic *E. coli* strain in the intestinal contents because ESBL producing enterobacteria have become a worldwide concern in poultry production [73]. The results of our study show synergistic effects on reducing the ex vivo survival of the *E. coli* model strain in crop contents compared to the control. The in vivo results on the quantification of the *Escherichia* group show a similar reducing synergism except for LA73/formulation C. Lactobacilli are known for their antagonistic activity against enterobacteria [74,75] and both probiotic strains showed exceptional inhibitory activity against the *E. coli* strain in vitro and ex vivo [38]. Contrary to these results, data from both the ex vivo assay and the *Escherichia* group quantification showed that the single addition of the probiotic strains had only slight effects on *E. coli* survival. Thus, the inhibitory activity of the phytobiotic products was probably necessary to enhance the impact of the

probiotic strains. However, the survival of the *E. coli* strain was also affected in the caecum, where active phytobiotic concentrations are considered low. This indicates that different modes of action may be in play.

In the context of ESBL producing enterobacteria and transfer of their resistance genes, the presence of the enterobacterial class 1 integron integrase 1 gene (*int1*) was also monitored. This enzyme is a key protein in the incorporation of foreign DNA in enterobacteria [76]. Its copy numbers correlated highly to the count of the *Escherichia* group ($p < 0.0001$; 0.551 coefficient) as well as to the count of enterobacteria ($p < 0.0001$; 0.423 coefficient). However, only the combination of LA73 and formulation L showed a reducing effect on *int*1 concentration. The *int1* gene is widely distributed in enterobacteria, and it is likely that certain enterobacterial species or strains responded differently to the supplementation of the feed additives. Nevertheless, the results clearly show that synergistic effects of probiotics and phytobiotics may be superior to a single addition to combat the spread of enterobacterial antibiotic resistance.

In the caecum, fermentation of undigested nutrients occurs [77], and the bacterial composition and activity is largely determined by incoming nutrients as well as bacteria from the small intestine [78]. Their metabolites (SCFA) can be used as an energy source by the host and may contribute to meet the energy requirements of the animal. Furthermore, the caecum also determines the output of the potentially detrimental bacteria into the environment and thus has an important impact on stable hygiene.

In this study, the impact of the feed additives on the caecal bacterial composition was much less pronounced compared to changes observed in the crop. This may point to the fact that the active compounds in the phytobiotic products (carvacrol, cinnamaldehyde, and eugenol) are metabolized in the small intestine. Consequently, their active concentration may be drastically reduced. Nevertheless, an antagonistic relationship between the abundance of a dominating unidentified *Clostridiales* genus and *Faecalibacterium* spp. was observed, as the significant reduction of *Faecalibacterium* spp. was always offset by a trend for an increase of the unidentified *Clostridiales* genus. As noticed for other parameters, a trend for synergistic effects was again visible for the combination LA73 and formulation L.

The dominating bacteria in the caecum are most likely to be the most prominent producers of SCFA from undigested nutrients, and indeed, an increase in the relative abundance of the unidentified *Clostridiales* genus always corresponded with increased acetate concentrations. The increased metabolite production points to an enhanced capacity to ferment undigested nutrients and indicates a more mature microbiota. As a mature microbiota is viewed as beneficial [79], the caecal microbiota, especially in animals, fed the combination LA73 and formulation L, could have developed faster than the microbiota in other feed groups.

There were two noteworthy exceptions to the generally low response of the caecal microbiota: a reduced colonization of *C. perfringens* and reduced ex vivo survival of the ESBL-producing *E. coli* in all treatment groups. Apparently, adverse conditions for these two detrimental species existed due to the addition of the feed additives. However, as the phytobiotic concentration is believed to be low in the caecum, these adverse conditions may have mainly originated from interbacterial competition or host-related responses that were induced in the crop or small intestine.

5. Conclusions

This study has shown that probiotics and phytobiotics can have beneficial synergistic effects on the intestinal microbiota in young chickens. The impact of the probiotics and phytobiotics was mainly confined to the crop, but synergistic effects were also observed in the caecum regarding the colonization of *C. perfringens* and the survival of an ESBL producing *E. coli* strain. Comprehensively considering the effects in microbiota shifts, changes in bacterial metabolites, and resilience to detrimental bacteria in host GIT, the combination of the *L. agilis* strain in combination with the formulation L was chosen as a synbiotic feed additive for large scale feeding trial on animal performance and health.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/7/12/684/s1, Table S1: Primer sequences and annealing temperatures for the detection of bacterial groups and species in the gut intestine, Table S2: The relative abundance [%] of bacterial phyla in the crop of 13-day-old broiler chickens fed different probiotics and phytobiotics, Table S3: The relative abundance [%] of bacterial phyla in the caecum of 13-day-old broiler chickens fed different probiotics and phytobiotics, Table S4: The relative abundance [%] of dominant bacterial order in the crop of 13-day-old broiler chickens fed different probiotics and phytobiotics, Table S5: The relative abundance [%] of dominant bacterial order in the caecum of 13-day-old broiler chickens fed different probiotics and phytobiotics, Table S6: The relative abundance [%] of dominant bacterial genera in the crop of 13-day-old broiler chickens fed different probiotics and phytobiotics, Table S7: The relative abundance [%] of dominant bacterial genera in the caecum of 13-day-old broiler chickens fed different probiotics and phytobiotics, Table S8: Ecological indices of the intestinal microbiota in the crop of 13-day-old broiler chickens fed different probiotics and phytobiotic, Table S9: Ecological indices of the intestinal microbiota in the caecum of 13-day-old broiler chickens fed different probiotics and phytobiotics, Table S10: 16S rDNA copy numbers of *Clostridium perfringens* in the intestinal tract of 13-day-old broiler chickens fed different probiotics and phytobiotics [log copy number 16S rDNA].

Author Contributions: W.V. and J.Z. conceived the original idea. H.R., W.V. and J.Z. organized the whole study. H.R., W.V., J.Z., E.-M.S. and F.G.B. designed the animal trial. H.R. and W.V. developed the experimental design. H.R., E.-M.S. and F.G.B. performed the animal experiments and sampling. H.R. performed the ex vivo experiment. H.R., T.D. and W.V. analyzed the data. H.R. wrote the first version of the manuscript. All authors revised the manuscript. All authors read and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION

Results for the establishment of the screening method, probiotic strain selection and characterization of probiotic candidates, formulation of probiotics and their *in vivo* activity in combination with phytobiotics have been presented and discussed in depth in **Chapter 4**, **Chapter 5** and **Chapter 6**.

Screening method for probiotic lactobacilli

The newly-developed screening method broadly followed existing techniques to select candidate probiotics by progressive reduction of candidate isolates with increasingly strict criteria. A major advancement of the method was the use of a specific target bacterium (ESBL *E. coli*) to assess the inhibitory activity of the probiotic candidates.

Compared with classic screening criteria, the developed three-staged method simplifies detection of inhibitory activity and massively enlarges of the number of isolates to be screened. Based on a literature review on probiotic selection, we found that the number of isolates ranged from 14 to 1150 isolates with the majority of publications using approximately 50 to 80 isolates (Robyn et al., 2012; Babot et al., 2014). The massive enlargement of isolate numbers leads to both high throughput and easy method for detection of inhibitory activity. Hence, a pre-screening was designed as the first step to quickly filter isolates by their inhibitory potential on microtiter plates. The advantage of liquid based growth inhibition assays over agar diffusion assays is their sensitivity and rapidity to observe subtle influences on growth (Fredua-Agyeman et al., 2017). In the first step, with over 7000 isolates subjected to the screening assay, only 76 isolates showed pH-dependent and/or pH-independent inhibitory potential. To more precisely study 76 selected isolates, lag time and lactate production were chosen as probiotic indicators due to their sensitivity and probable probiotic factors (Swinnen et al., 2004; Rufián-Henares and Morales, 2008). In the end, a total of five isolates among all 76 isolates that exhibited the highest indices for both parameters were chosen.

In view of the complex intestinal environment, *in vitro* models cannot reflect antibacterial effects that may occur in the animal, because it is always questionable whether probiotics maintain their inhibitory activity *in vivo* (Talpur et al., 2012). Consequently, *in vitro* tests cannot mimic the complex intestinal matrix and truly reflect the inhibitory activity of probiotics in the GIT of animals (Saint-Cyr et al., 2016). Thus, an *ex vivo* model was established based on the work of Starke and coworkers, to find a compromise between *in vitro* and *in vivo* approach (Starke et al., 2013). This *ex vivo* model mimics a maximum of *in vivo* conditions with cost-efficiency and ease of handling of *in vitro* models. From this novel model, two *Lactobacillus* candidates, one *L. salivarius* and one *L. agilis* strain, were eventually selected. Although host-related gastric stresses were not considered as main criteria, resilience against the acid, bile and osmotic

stress in accordance of the physiological conditions in the poultry GIT were determined. Both candidates were highly tolerant against putative gastric stress. Safety assessment is an important approach before their application on animals. In terms of safety check for lactobacilli species, a major concern is their antibiotic resistance (Imperial and Ibana, 2016). Hence, the resistance profile of selected candidates was assessed on the basis of commonly used antibiotics. Both final candidates (*L. salivarius* and *L. agilis*) demonstrated no resistance against recommended antibiotics in human medicine, which increased the confidence for a further development as safe feed additive in the future. The first part of the thesis successfully validated a three-staged method to massively screen potential probiotic isolates for pathogenic targets with clear biomarkers. Moreover, the selected candidate probiotics were shown to be suitable as feed additive in poultry nutrition.

Production of probiotics on the laboratory scale

Probiotic traits in bacterial strains do not necessarily mean that they can be successfully applied in practice. The efficiency of probiotic products is highly dependent on cell viability, since the mode of action of probiotics is conferred by living cells (Del Piano et al., 2006). Thus, the optimization of production parameters of a probiotic is a prerequisite for a successful probiotic product during fermentation and stability throughout processing and storage until delivery to the animal. The metabolic fingerprints of two probiotic candidates (*L. salivarius* and *L. agilis*) were analyzed with BIOLOG AN® plate (Stefanowicz, 2006) and indicated that different substrates were preferred by the strains. Six top substrates were selected to supplement the basal medium MRS for probiotic biomass production to study a possible booster effect on bacterial growth. The results indicated that the supplementation of sucrose for *L. salivarius* and mannose for *L. agilis* shortened the exponential growth phase and yielded more biomass than with MRS alone.

Lactobacillus spp. are facultative anaerobes, but several species do not tolerate oxygen well (Mitropoulou et al., 2013; Maresca et al., 2018). It is known that aerobic cultivation requires less energy and is thus more economic (Siciliano et al., 2019). Therefore, probiotics with high oxygen tolerance and capability to maintain robust growth under aerobic condition are preferred for a scale-up production. Both probiotic strains were able to grow well in aerobic condition, leading to a more economic biomass production for those strains.

Most probiotics are not used directly as fresh cultures in animal nutrition, as many strains loose viability in this form. Freeze drying and spray drying are usual processes to formulate probiotics (Broeckx et al., 2016). However, even freeze drying causes a viability loss of probiotic cultures. Thus, the protection of the probiotic cells during processing is of high importance. Numerous investigations reported that cryo-protective mixtures are able to yield synergistic effects on cell viability. Yet, results from various studies indicate that protectants work in a strain-specific

manner. Hence, three potential protective substances were studied in the second part of the present thesis, namely skim milk, sucrose, and trehalose. For a better understanding of the interactions among the three factors and minimizing data bias, the response surface method strategy with Box-Behnken Design was assigned in this work. Similar to some other studies, skim milk was assumed to be the most influencing protective factor (Lu et al., 2017). Sucrose and trehalose demonstrated a synergistic protective effect as they enhanced the protection offered by skim milk. This effect was in accordance with a study on preserving *Candida sake* cultures (Abadias et al., 2001). On the basis of a model built by the interaction of the three factors, an optimized protective composition was generated (0.14 g/L skim milk, 0.08 g/L and 0.09 g/L trehalose for *L. salivarius*, and 0.15 g/L skim milk, 0.08 g/L, and 0.07 g/L trehalose for *L. agilis*, respectively). These formulations were also determined with their storage stability. Although storage temperature was the overriding effector, the protective formulations endowed improved stability to both probiotics strain. In this part of the thesis, production parameters were optimized specifically for two selected probiotic candidates that were selected for a feeding trial due to their exceptional probiotic activity.

Synergistic effects of pro- and phytobiotics in a feeding trial

Probiotics and phytobiotics have been extensively investigated as feed additives individually in the past decades, yet there is a lack of in depth investigations on the combination of these two types of feed additives. Therefore, the third part of this thesis was concerned with the possible synergistic effects of pro- and phytobiotics in a feeding trial with broiler chickens. This part investigated the effects of different feed additives on the gut microbiota of young broilers. It compared the effects of single use of probiotic/ phytobiotic versus their respective combinations to investigate possible synergistic effects. The rationale behind the selection of young broilers as research objective was that young animals are still in the process of developing a mature microbiota. This process may thus respond more sensitively to alterations by feed additives (Kers et al., 2018b; Rubio, 2018). The detailed modulation on the microbiota composition and –activity is shown and discussed in Chapter 6.

The crop and caecum of birds are viewed as the pre- and post-gastric fermentation chambers, which defines the input and output of GIT bacteria (Lu et al., 2003; Gallazzi et al., 2008; Ercolini and Fogliano, 2018). However, as expected, the microbiota in crop and caecum responded differently to the feed additives. Generally speaking, the effects of the pro- and phytobiotics and their combinations were more pronounced in the crop than in the caecum. This outcome could be possibly due to the phytogenic products (carvacrol, cinnamaldehyde and eugenol) which are absorbed in the small intestine and consequently their active concentration may be drastically reduced in the caecum. This indicates that the micro-ecology of different intestinal sections must be viewed individually. A notable synergistic effect in this feeding trial was that

the additives modified *Lactobacillus* spp. populations, as *L. salivarius* in combination with formulation L as well as *L. agilis* with formulation C significantly elevated *Lactobacillus* spp. abundance. To our best knowledge, this is the first report about the promotion of *Lactobacillus* by a synergistic effect of probiotics and phytobiotics. A similar effect was observed in the animals fed with the single use of probiotic or phytobiotic (Gheisar et al., 2015; Gao et al., 2017). The abundance of *Clostridium sensu stricto* 1 was high in single probiotic and formulation L supplemented feed groups, but was dramatically reduced in combinations of LS1 with both phytobiotic products and especially in LA73 with formulation L. These results signify again that the synergistic mode of action on certain bacteria is not additive but relies on the impact of the feed additives on other bacteria. As to the bacterial metabolites, lactate was significantly and acetate was numerically lower in the crop of groups with single phytobiotic addition. Although the active ingredients like cinnamaldehyde or eugenol show high antibacterial activity *in vitro*, the observed reduction of bacterial activity without significant reduction of absolute counts of bacteria may be viewed as a bacteriostatic effect. Consequently, at the employed in-feed concentrations, the phytogenic products may not inhibit total bacterial growth *per se*, but significantly reduce their activity in the crop. Interestingly, certain combinations, for instance, formulation L in combination with LA73, reversed the inhibition of lactate production, showing significantly or numerically higher concentrations of metabolites.

Extended-Spectrum-Beta-Lactamase (ESBL) producing enterobacteria have become a worldwide concern in poultry production (Saliu et al., 2017). Thus, we also evaluated whether the feed additives may decrease the colonization of ESBL producing *E. coli* in the birds. We therefore estimated the *ex vivo* survival of an ESBL *E. coli* model strain in crop- and caecum contents. Data from both *ex vivo* assay and *Escherichia* group quantification suggested that the single addition of the probiotic strains had only slight effects on *E. coli* survival, but in combination with phytobiotics, some feed groups showed a significant reduction of the *E. coli* survival in *ex vivo* assays. Thus, the inhibitory activity of the phytobiotic products was probably necessary to enhance the impact of the probiotic strains in the crop. However, the inhibition by synergistic combinations in the caecum may follow a different mode of action, as phytobiotics may be absorbed in the foregut. To further understand the resilience against colonization against ESBL enterobacteria, the presence of the enterobacterial class 1 integron integrase 1 gene (*int1*), which is an essential element in the incorporation of foreign DNA in enterobacteria was also monitored (Fluit and Schmitz, 1999). The only decrease of *int1* genes was observed in the combination group of LA73 and formulation L, suggesting a synergistic effect on subsequently reducing the ESBL gene transmission. Nevertheless, the results clearly show that synergistic effects of pro- and phytobiotics may be superior to single addition to combat the spread of enterobacterial antibiotic resistance. Additionally, the colonization of *C.*

perfringens was also reduced in birds receiving combinations of feed additives. In general, the results of this section of the thesis confirmed that synergistic effects of combination of probiotics and phytobiotics in terms of gut microbiota composition and –activity as well as inhibition against certain detrimental bacteria. This was especially pronounced for the combination of LA73 in combination with phytobiotic formulation L.

To conclude, the present thesis firstly established a high-throughput method to quickly screen possible probiotic isolates against a specific target bacterium with a clear biomarker (ESBL producing *E. coli* in our study) and showed the potential of the method to be extended to a broader spectrum of probiotics with only minor modifications of the screening criteria. With this method, two *Lactobacillus* strains with activity against ESBL *E. coli* were selected from more than 7000 isolates.

These strains were then optimized for their production parameters. The best condition of cultivation and formulation was determined and probiotic powders with high residual viability were formulated.

The probiotic strains were then tested on young birds with or without commercial phytobiotics. The impact of the pro- and phytobiotics were mainly confined to the crop, but synergistic effects were also observed in the caecum regarding the colonization of *C. perfringens* and the survival of an ESBL producing *E. coli* strain.

Thus, in this thesis, both probiotics and phytobiotics showed capability to exert the beneficial modification of the gut microbiota and potential to promote host health, while the combination of *L. agilis* and formulation L is preferred due to its superior synergistic effects on important bacterial parameters.

CHAPTER 8: SUMMARY

Selection, Preparation and Application of Host-derived Probiotic Lactobacilli from Chicken and their Synergistic Effects with Phytobiotics on Broilers.

In **Chapter 1**, the general scientific rationale of the current thesis is introduced. In-feed antibiotics have benefited the animal husbandry for over 80 years, but antibiotic resistance emerged as a massive problem in recent decades. Antibiotics have been broadly used as growth promoters and for veterinary treatments in animal industry. This has contributed to a fast spread of antibiotic resistance, especially in poultry. Thus, it calls for theoretical and practical efforts to select promising alternatives to in-feed antibiotics.

Several alternative feed additives have been comprehensively studied, among them probiotics and phytobiotics. However, the limitation in host-specificity of probiotics and their combination with phytobiotics in animal nutrition has restricted the in-depth understanding towards their functionality and role as AGP alternative. Thus, the refinements in probiotic screening, formulation and application is necessary and meaningful to better substitute AGP in animal production.

In **Chapter 2**, a literature review regarding the research advances of in-feed probiotics and phytobiotics is provided. The literature review summarizes both probiotics and phytobiotics as AGP alternatives from the aspect of growth promotion, GIT microbiome alteration, immuno-regulation, practical formulation and their respective combinations with other additives. The main objectives of the present thesis are to develop a rapid and efficient protocol of probiotic screening and preparation and investigate possible beneficial synergistic effects with phytobiotics.

In **Chapter 3**, on basis of the given background, the aim of this study was to isolate and produce host-specific probiotics and to investigate the possible synergistic effects of these probiotics with commercial phytobiotics. This chapter also clarified the research objectives of the thesis including establishment of new screening method, *in vitro* characterization, optimization of production and stabilization and a proof-of-principle by an *in vivo* evaluation.

The **Chapters 4** to **6** comprehensively summarize investigations on the establishment of the novel screening method, production of probiotics and their effects of bacterial development in young broiler chickens. With the three-staged screening method, over 7000 isolates were filtered through the screening and finally two candidates were selected for their excellent *in vitro* and *ex vivo* activity against ESBL *E. coli*. The strain-specific production parameters of two probiotic strains were optimized regarding cultivation and formulation, achieving both relative high yields and high stability after production. The chosen probiotic strains and two commercial phytobiotics as well as their combinations were tested in a proof-of-principle feeding trial. The composition and activity of crop and caecum microbiota of young broilers were analyzed to demonstrate the impact of the feed additives on the host intestinal ecology. Additionally, the

ability to inhibit colonization of ESBL producing *E. coli* as well as *C. perfringens* in either foregut or hindgut of birds was documented. These three consecutive studies showed an overarching process from isolation of probiotic strains to functional feed additives in a feeding trial.

In **Chapter 7**, the results from the current thesis are discussed in general. The efficiency of the three-staged method was emphasized by discussing handling and financial cost during the screening step. The method also allows an easier enlargement of the screening scale compared to traditional one-step screening procedures. It also endows the feasibility to drastically enlarge the screening scale compared to traditional one-step screening methods. With both experimental and computational means, the formulation of selected probiotic strains into feed additives was improved. Finally, the prepared probiotic feed additives were shown to exert beneficial effects on the gut microbiota of young broilers. This was especially observed for a specific combination of probiotic and phytobiotic. This evidenced the great potential of probiotics in combination with phytobiotics as an alternative to the use of AGP in broiler husbandry.

KAPITEL 9: ZUSAMMENFASSUNG

Auswahl, Herstellung und Anwendung von aus dem Wirt stammenden probiotischen Lactobacillen von Hühnern und ihre synergistischen Wirkungen mit Phytobiotika bei Broilern.

In **Kapitel 1** wird die allgemeine wissenschaftliche Begründung der vorliegenden Arbeit vorgestellt. Antibiotika haben die Tierhaltung seit über 80 Jahren begünstigt, aber Antibiotikaresistenzen haben sich in den letzten Jahrzehnten zu einem massiven Problem entwickelt. Antibiotika wurden in großem Umfang als Leistungsförderer und zur Prophylaxe und Therapie in der Tierhaltung verwendet. Dieses hat zu einer schnellen Ausbreitung von Antibiotikaresistenzen beigetragen, insbesondere bei Geflügel. Daher sind theoretische und praktische Anstrengungen erforderlich, um vielversprechende Alternativen zu Antibiotika in Futtermitteln auszuwählen.

Mehrere alternative Futtermittelzusätze wurden umfassend untersucht, darunter Probiotika und Phytobiotika. Die Einschränkung der Wirtsspezifität von Probiotika und ihre Kombination mit Phytobiotika in der Tierernährung hat jedoch das eingehende Verständnis der Funktionalität und Rolle als AGP-Alternative eingeschränkt. Daher ist die Verfeinerung des Screenings, der Formulierung und der Anwendung notwendig und sinnvoll, um AGP in der Tierproduktion besser zu substituieren.

In **Kapitel 2** wird eine Literaturübersicht zu den wissenschaftlichen Fortschritten bei Probiotika und Phytobiotika gegeben. Die Literaturübersicht fasst sowohl Probiotika als auch Phytobiotika als AGP-Alternativen unter den Gesichtspunkten Leistungsförderung, Mikrobiom des Gastrointestinaltrakts, Effekte auf das Immunsystem, praktische Formulierung unter Berücksichtigung von Kombinationen und der Interaktion mit anderen Additiven. Das Hauptziel der vorliegenden Arbeit war es, ein effizientes Protokoll für das Screening von potenziellen probiotischen Bakterien, Verfahren für die Herstellung zu entwickeln und mögliche vorteilhafte synergistische Effekte mit Phytobiotika zu untersuchen.

In **Kapitel 3** wurde anhand des gegebenen Hintergrunds das Ziel dieser Studie verfolgt, wirtsspezifische Probiotika zu isolieren und zu produzieren und die möglichen synergistischen Wirkungen dieser Probiotika mit kommerziellen Phytobiotika zu untersuchen. In diesem Kapitel wurden auch die Forschungsziele der Dissertation geklärt, einschließlich der Etablierung einer neuen Screening-Methode, der In-vitro-Charakterisierung, der Optimierung der Produktion und der Stabilisierung sowie einer In-vivo-Bewertung.

In den **Kapiteln 4** bis **6** werden Untersuchungen zur Etablierung der neuartigen Screening-Routine, zur Herstellung stabiler Probiotika und zu deren Auswirkungen auf die Entwicklung der intestinale Mikrobiota bei jungen Masthühnern umfassend zusammengefasst. Mit einem dreistufigen Screening-Verfahren wurden über 7000 Isolate geprüft und schließlich zwei Kandidaten aufgrund ihrer erwünschten probiotischen Aktivität *in vitro* und *ex vivo* ausgewählt. Die stammspezifischen Produktionsparameter wurden hinsichtlich Kultivierung und Formulierung optimiert, wobei sowohl eine relativ hohe Ausbeute als auch eine hohe Stabilität nach der Produktion erzielt wurden. Die ausgewählten probiotischen Stämme und zwei handelsübliche Phytobiotika sowie deren Kombinationen wurden in einem Fütterungsversuch getestet. Die Zusammensetzung und Aktivität der Kropf- und Caecum-Mikrobiota junger Masthähnchen wurde analysiert, um den Einfluss der Futterzusätze auf die Darmökologie des Wirts nachzuweisen. Zusätzlich wurde die Fähigkeit dokumentiert, die Kolonisierung von ESBL-produzierenden *E. coli* sowie *C. perfringens* im vorderen und hinteren Verdauungstrakt zu hemmen. Diese drei aufeinanderfolgenden Studien zeigten in einem Fütterungsversuch einen übergreifenden Prozess von der Isolierung probiotischer Stämme bis hin zu funktionellen Futterzusatzstoffen.

In Kapitel 7 werden die Ergebnisse der aktuellen Arbeit übergreifend diskutiert. Die Effizienz der dreistufigen Methode wurde durch die Diskussion der Handhabung und der Kosten während des Selektionsverfahrens unterstrichen. Das Verfahren ermöglicht auch eine einfachere Ausweitung des Testprinzips im Vergleich zu herkömmlichen einstufigen Screening-Verfahren. Es macht es möglich, den Umfang der Untersuchungen im Vergleich zum herkömmlichen einstufigen Screening erheblich zu vergrößern. Sowohl experimentell als

auch unter Nutzung rechnerischer Verfahren wurde die Formulierung ausgewählter probiotischer Stämme zu praktisch einsetzbaren Futtermittelzusatzstoffen verbessert. Schließlich wurde gezeigt, dass die hergestellten probiotischen Futterzusätze positive Auswirkungen auf die Darmmikrobiota junger Broiler haben. Dies wurde insbesondere für eine spezifische Kombination von Probiotika und Phytobiotika beobachtet. Dies belegt das große Potenzial von Probiotika in Kombination mit Phytobiotika als Alternative zum Einsatz von AGP in der Masthühnerhaltung.

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Hiermit erkläre ich an Eides statt, die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

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