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The phyllosphere microbiome of medicinal plants

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Abstract

In the course of this work, the phyllosphere microbiome of medicinal plants was analyzed, thereby putting a special focus on probiotic lactic acid bacteria (LAB). LAB are known for their health promoting effects when ingested and play an important role in the development, preservation as well as regeneration of a healthy microbiome. Besides their occurrence as an essential part of the human microbiome (e.g. intestinales) they are also inhabiting dairy products and plant material, particularly fermented foods. Therefore, the diversity and composition of the (lactic acid) bacterial microbiome of different medicinal plants was investigated through fermentation of leaves of *Calendula officinalis* L. and *Matricaria chamomilla* L. in comparison to *Brassica oleracea* L. var. *capitata*. Bacterial isolation from six weeks fermented plant material yielded 394 potential LAB isolates which could be classified by amplified ribosomal RNA gene restriction analysis (ARDRA) into five distinct groups. A representative set of isolates covering all groups was sequenced by partial 16S rRNA gene sequencing, confirming their affiliation to the LAB, namely to the genera *Enterococcus* and *Lactobacillus*. Cultivation-independent analyses were used to investigate the natural ecto- and endospheric phyllosphere microbiome as well as that of fermented plant material during the fermentation period. Illumina-based amplicon sequencing of 16S rRNA genes unveiled a distinct phyllosphere microbiome in the starting samples, while the endosphere revealed a high similarity. During fermentation, significant microbial shifts were observed, whereby LAB were enhanced in all approaches but never dominated. The natural leaf microbiome and the indigenous LAB communities of field-grown *Asteraceae* medicinal plants thereby showed plant-specificity and habitat-specificity. Besides the leaf surfaces, also the leaf endosphere was identified as source for biopreservative LAB. Molecular fingerprints using single strand conformational polymorphism (SSCP) analysis of 16S rRNA genes showed high similarity of the fungal communities of the various fresh cabbage samples whereas some variation in the band patterns of the different medicinal plants was ascertained. In contrast, the opposite was true for the the *Ascomycota* community, whose fingerprints were almost identical for the medicinal plants but showed high variation between the cabbage samples. Colonization patterns examined through fluorescence *in situ* hybridization (FISH) in combination with confocal laser scanning microscopy (CLSM) led to the detection of *Gammaproteobacteria* on unfermented *C. officinalis* L. blossoms whereas on the fermented plant samples only taxonomically not further characterized *Eubacteria* could be visualized. During the fermentation process, bacterial diversity decreased showing that LAB are able to

suppress other (pathogenic) microorganisms, an observation already noticed and used by our ancestors. This circumstance and the fact that LAB are naturally inhabiting medicinal plants and that they can be enriched within fermentation broadens the spectrum of traditional healing applications of the plants used for medicinal purposes. Moreover, it opens possibilities of natural future applications, not only in the herbal- and academic medicine but also in the food industry and the agricultural sector.

Another part of the thesis was the genome sequencing of three promising isolates already known for their potential as plant growth promoting bacteria. Thereby, the complete genome sequence of *Bacillus amyloliquefaciens* Co1-6 and the draft genome sequences of *Paenibacillus polymyxa* Mc5Re-14 and *Streptomyces* sp. Wb2n-11 were sequenced and annotated.

Furthermore, rep-PCR fingerprinting (BOX-PCR) of selected antagonistic strains isolated from native Egyptian desert soil as well as cultivated Egyptian agriculture land (rhizosphere of medicinal plants) was implemented. It was shown that some endorhizal antagonists, although isolated from different plants (*M. chamomilla* L. and *C. officinalis* L.), shared a similarity of over 95% in their genetic fingerprints.

Kurzfassung

Die vorliegende Arbeit erforschte das Phyllosphären-Mikrobiom von Heilpflanzen, wobei der Fokus auf die Untersuchung von Milchsäurebakterien gelegt wurde. Die gesundheitsfördernde Wirkung nach der Zufuhr von Milchsäurebakterien ist schon lange bekannt, zumal sie eine wichtige Rolle in der Entwicklung, Erhaltung ebenso wie der Regeneration eines gesunden Mikrobioms einnehmen. Neben ihrer Bedeutung als ein wichtiger Bestandteil des menschlichen Mikrobioms (z.B. Darm) kommen sie auch in Milchprodukten sowie auf Pflanzenmaterial vor, vor allem jedoch in Lebensmitteln, die fermentiert wurden. Im Zuge dieser Arbeit wurde daher die Diversität und Zusammensetzung des (Milchsäurebakterien-) Mikrobioms von verschiedenen Heilpflanzen (*Calendula officinalis* L. und *Matricaria chamomilla* L.) im Vergleich zu Weißkraut (*Brassica oleracea* L. var. *capitata*) während ihrer Fermentation untersucht. Die Isolation von Bakterien aus für sechs Wochen fermentiertem Pflanzenmaterial führte zur Gewinnung von 394 potenziellen Milchsäurebakterien-Isolaten, welche durch ribosomale RNA Gen-Restriktionsanalyse (ARDRA) in fünf Gruppen unterteilt werden konnten. Partielle 16S rRNA-Gen-Sequenzierung ausgewählter Isolate aller Gruppen bestätigte deren Zugehörigkeit zu den Milchsäurebakterien, nämlich zu den Gattungen *Enterococcus* und *Lactobacillus*. Mit Hilfe kultivierungsunabhängiger Analysen wurde das natürliche ecto- und endosphärische Phyllosphären-Mikrobiom sowie jenes von fermentiertem Pflanzenmaterial im Laufe der Fermentation untersucht. Eine Illumina-basierte Amplikon-Sequenzierung der 16S rRNA Gene zeigte ein unterschiedliches Phyllosphären-Mikrobiom der beiden unfermentierten Pflanzen, wohingegen eine hohe Ähnlichkeit der Endosphärenbesiedelung festgestellt werden konnte. Während der Fermentation zeigte sich eine signifikante mikrobielle Änderung, welche durch eine Zunahme aber keine Dominanz von Milchsäurebakterien in allen Versuchsreihen geprägt war. Das natürliche Blattmikrobiom und die indigene Milchsäurebakteriengemeinschaft wiesen dabei eine Pflanzenspezifität ebenso wie eine Spezifität gegenüber dem Habitat auf. Zusätzlich zur Blattoberfläche konnten Milchsäurebakterien dabei auch in den Blattendosphären nachgewiesen werden. Molekulare Fingerprints durch Einzelstrang-Konformationspolymorphismus (SSCP)-Analyse der 16S rRNA Gene zeigten eine höhere Ähnlichkeit unter den pilzlichen Gemeinschaften der unterschiedlichen, unfermentierten Krautproben, wohingegen die Bandenmuster der verschiedenen Heilpflanzen eine gewisse Varianz aufwiesen. Im Gegensatz dazu waren die Fingerprints der Ascomyceten der unterschiedlichen Krautproben untereinander sehr divers, zeigten jedoch innerhalb der Heilpflanzen nur wenige Unterschiede. Fluoreszenz *in situ*

Hybridisierung (FISH) in Kombination mit konfokaler Laserscanning-Mikroskopie (CLSM) ermöglichte eine Detektion von Gammaproteobakterien an den unfermentierten Blüten der Ringelblume, wohingegen auf fermentiertem Pflanzenmaterial nur taxonomisch nicht weiter charakterisierte Eubakterien detektiert werden konnten. Während des Fermentationsprozesses sank die bakterielle Diversität, was zeigte, dass Milchsäurebakterien die Fähigkeit besitzen, das Wachstum von anderen (pathogenen) Bakterien zu reduzieren – eine Beobachtung, die bereits unsere Vorfahren schon für sich nutzten. Dieser Umstand und die Tatsache, dass Milchsäurebakterien natürlich in und auf Heilpflanzen zu finden sind und dabei durch Fermentation angereichert werden können, erweitert das Anwendungsspektrum der Heilpflanzen für medizinische Zwecke. Darüber hinaus eröffnet es auch Möglichkeiten, diese natürlich vorkommende Pflanzen-Bakteriengemeinschaft nicht nur in der naturkundlichen- und Schulmedizin sondern auch in der Lebensmittel- und Agrarindustrie einzusetzen.

Einen weiteren Teil dieser Arbeit umfasste die Sequenzierung von drei Bakterienstämmen (*Paenibacillus polymyxa* Mc5Re-14, *Bacillus amyloliquefaciens* Co1-6 und *Streptomyces* sp. Wb2n-11), welche aufgrund ihrer bereits bekannten pflanzenwachstumsfördernden Eigenschaften ausgewählt wurden. Dabei konnten die Genomsequenzen aller drei Stämme sequenziert und annotiert werden.

Weiters wurde eine rep-PCR-Fingerprint-Analyse (BOX-PCR) von antagonistischen Bakterienisolaten aus nativem, ägyptischen Wüstenboden sowie landwirtschaftlich genutztem Wüstenboden (Rhizosphäre von Arzneipflanzen) durchgeführt. Es konnte gezeigt werden, dass Antagonisten der Endorhiza, obwohl sie von unterschiedlichen Pflanzen (*M. chamomilla* L. und *C. officinalis* L.) gewonnen wurden, eine hohe Ähnlichkeit (teilweise >95%) in ihren genetischen Fingerprints aufwiesen.

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List of Contents

Introduction

Correlation of the human gut microbiome, lactic acid bacteria, probiotics and biocontrol

Known as the habitat with the highest bacteria density, the gut is said to be the most densely populated microbial ecosystem on Earth. From the human oral cavity to the rectum a microbial population with nearly 800 species is found, unique from individual to individual (Albesharat *et al.*, 2011; Selber-Hnatiw *et al.*, 2017; Hillman *et al.*, 2017). With a cell concentration of up to 10^{12} bacteria per gram in human feces the number of bacterial cells is as much as 10 times higher than the number of tissue cells forming the human body (Heczko *et al.*, 2006). The gut microbiome in the digestive tract is standing in a symbiotic relationship with its human host supporting nutritional, physiologic and protective processes. The production of vitamins like biotin and vitamin K, the digestion of hard-to-digest polysaccharides in plant foods, the evolution of the immune system as well as the protection against pathogens are a result of that interaction. The functioning of this synergism is based on a balanced equilibrium, where changes in the composition of the microbes can lead to health disorders such as irritable bowel syndrome, Crohn's Disease, obesity and weight loss (Devirgiliis *et al.*, 2011; Balamurugan *et al.*, 2014). In our fast pace of life however, a healthy lifestyle with a balanced diet is often neglected by just grabbing a quick snack. This is also true for Asia, known for its healthy diet, where a shift from traditional food towards commercial fast food in combination with urbanization has led to a decline in the consumption of fermented aliments (Tamang *et al.*, 2016). The colonization of the sterile digestive system starts at the time of birth with facultative anaerobic pioneer bacteria like *Staphylococcus*, *Enterococcus*, *Streptococcus* spp. and members of the *Enterobacteriaceae*. Due to oxygen depletion, the facultative anaerobes are replaced by obligate anaerobes like *Bifidobacterium*, *Bacteroidetes* and *Clostridia*. The gastrointestinal tract thereby changes until the age of 2-3 years, starting from a relatively low diversity until the establishment of a highly diverse and rather stable ecosystem (Chassard *et al.*, 2014). In adulthood, factors such as secretory products, peristalsis, food transit time, host genotype as well as environmental factors such as type of diet or the direct ingestion of living microorganisms can have an influence on the microbial community structure (Albesharat *et al.*, 2011). Therefore, a varied

nutrition is not only important to get enough vitamins and nutrients, but plays also an important role for the development of a healthy microbiome. In this regard, probiotics display the possibility to prevent a so called dysbiosis – a disturbance of the intestinal flora – especially lactic acid bacteria are at the focus of bringing a health benefit when obtained through our diet.

As one of the first, Metchnikoff theorized already over 100 years ago that "*there is a dependence of the intestinal microbes on food*" and that these microorganisms are able to "*modify the flora of our bodies and to replace the harmful microbes by useful microbes*". He stated even back then that especially lactic acid bacteria do have a positive effect when ingested: "*A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes, as the general belief is that microbes are all harmful. This belief, however, is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honourable place*" (Metchnikoff, 1908). Lactic acid bacteria of the phylum *Firmicutes* are widely distributed, colonizing habitats where rich, carbohydrate-containing substances are available which they ferment in homo- or heterofermentative ways, mainly into lactic acid. This production of lactic acid made them an important tool to preserve food since ancient time. The application of lactic acid bacteria in the food industry has also established because they are nontoxic to human as they are a part of the natural intestinal flora. Nowadays, especially the genus *Lactobacillus* – a non-spore forming, aerotolerant, rod-shaped, Gram-positive bacterium – is used in preserving food due to its antimicrobial properties caused by the production of organic acids, other organic compounds, hydrogen peroxide and bacteriocins. Their use as probiotics is moreover linked to their putative health promoting effects (Bernardeau *et al.*, 2008; Von Wright *et al.*, 2012). One common definition of probiotics can be attributed to an expert consultation on the evaluation of health and nutritional properties of probiotics in food of the World Health Organisation (WHO) and the Food and Agricultural Organization of the United Nations (FAO) who defined probiotics as "*Live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2001). Health benefits include cancer prevention, the regulation of cell proliferation and apoptosis (Di Luccia *et al.*, 2013). Studies reported an antagonistic potential of LAB due to their antimicrobial activities against human pathogens such as *Staphylococcus aureus*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus epidermidis*, *Propionibacterium acnes* and *Escherichia coli* (Bernet-Camard

et al., 1997; Darsanaki *et al.*, 2012; Shokryazdan *et al.*, 2014). Through the improvement of the immune response, probiotics are involved in reducing the onset of systemic inflammatory induced diabetes. Effects on the serum cholesterol were demonstrated in studies as well as the ability of lactic acid bacteria to inhibit micelle formation due to the deconjugation of bile salts in the intestine. An enhancement of the lipid metabolism was observed *in vivo* through the reduction of lipid peroxidation. It is moreover presumed that the composition of the gut microbiome is associated with the promotion of obesity in children. The impact of probiotics on the human body can thereby only be achieved when the bacteria survive the gastric juices and bile of the human gut and are then – after digestion – able to colonize the digestive tract (Aggarwal *et al.*, 2013).

Aside their function as probiotics and the promotion of human health, lactic acid bacteria do also play a role in agriculture, promoting plant health through their antagonistic potential against several plant pathogens. The disadvantage of chemical means which are considered to be toxic and the fact that some of them cannot be used for postharvest treatment in agriculture, the rising resistance of some pathogens against the chemicals and also the high costs of the development of new chemicals have led to a higher acceptance of microorganism-based treatment methods. Antagonistic potential of lactic acid bacteria has been detected for several plant pathogens, e.g. *Botrytis cinerea*, *Pectobacterium carotovorum*, *Monilinia laxa* and *Xanthomonas campestris* (Trias *et al.*, 2008).

Medicinal plants and cabbage – sources of plant material for colonization analysis and fermentation studies

Matricaria chamomilla L.

The german chamomile – *Matricaria chamomilla* L. (Syn. *Matricaria recutita* L.) attributed to the family of *Asteraceae* is one of the oldest and most popular medicinal herbs used by mankind. The applications of alimental, cosmetic and pharmaceutical use are based on tea, ointments, tinctures and herbal extracts. Since ancient time chamomile is promised to relieve several diseases due to its antiinflammatory, analgesic, sedative, antimicrobial, antiallergic, antihyperglycemia and antispasmodic effects. The annual plant with its white chamomile flower heads reaches a high of 20-50 cm and its medicinal benefits result from its characteristic blue coloured oil (essential oil), flavonoids and polysaccharides (Ganzera *et al*., 2006; Haghi *et al*., 2014; Srivastava *et al*., 2010). As secondary metabolites, over 120 chemical constituents have been identified in chamomile (Singh *et al*., 2011), whereby the main components regarding the flavonoids are apigenin, quercetin, patuletin, luteolin, while in the volatile, essential oil the terpenoids α-bisabolol and its oxides and azulenes, including chamazulene are predominant. Apigenin is in the focus of science due to influencing cellular processes ranging from the inflammatory response, the regulation of cell membrane transport, the cytokine production even to cell cycle progression. Due to the inhibition of enzymes involved in intracellular signalling cascades and the reduced expression of proto-oncogenes, apigenin is discussed to have a potential anticancer effect (McKay & Blumberg, 2006). Bisabolol and chamazulene possess antiseptic effects, bisabolol moreover is antimicrobial and antiinflammatory, which makes it a preferred component in cosmetic products (Singh *et al*., 2011).

Calendula officinalis L.

Pot marigold (*Calendula officinalis* L.) of the family of *Asteraceae* is native to Egypt and the Mediterranean region and used as medicinal plant for centuries. The flower with its bright or yellow orange flower heads reaches a high of 30-60 cm. Marigold is known for its antiinflammatory, antibacterial, antifungal, anti-HIV, spasmolytic, antioedematous, anticancer, antioxidant, antiseptic, antiviral and hepatoprotective properties. Applied as infusion, tincture, fluid extract or ointment it can stimulate granulation and promote the

healing of skin wounds resulting from bruises, cuts and burns. Apart from the improved cicatrization, marigold can prevent the spread of infections. Constituents responsible for those properties are the containing volatile oil, flavonoids, terpenoids, carotenoids, coumarins, quinones, carbohydrates, lipids, amino acids, as well as other minor constituents (Khalid & Teixeira da Silva, 2012).

Brassica oleracea L.

White cabbage (*Brassica oleracea* L. var. *capitata*) is beside the genus cauliflower, broccoli, Brussels sprouts and kale a member of the family of *Brassicaceae* and among them one of the most popular *Brassicaceae* consumed in Europe. *Brassicaceae* are renowned for their substantial number of polyphenolic compounds which are known for their antioxidative properties acting as protective factors against cancer and heart diseases. Beside the polyphenols, cabbage contains other phytochemicals like carotenoids, vitamin C and glucosinolates (Podsędek, 2007; Šamec *et al*., 2014). Glucosinolates are hydrolyzed by the enzyme myrosinase after plant cell disruption, which turns the biologically inactive form of glucosinolates into breakdown products, partly responsible for the cancer-protecting effects (Peñas *et al*., 2010). Beside the bioactivity of glucosinolate breakdown products, they are also responsible for the typical flavor of *Brassica* vegetables (Fenwick *et al*., 1983). In addition to the bioactive compounds, the main constituents of white cabbage are carbohydrates, which amount to nearly 90% dry weight in cabbage. One third thereof are dietary fibers, the other two thirds being low-molecular-weight carbohydrates. Cabbage can be eaten raw or prepared in boiled or fermented ways (Wennberg *et al*., 2006). An important product is sauerkraut (shredded and salted cabbage) resulting from the spontaneous fermentation performed by lactic acid bacteria occurring naturally on the leaves of cabbage (Peñas *et al*., 2010).

Sekem – sampling site managed under organic (biodynamic) agriculture conditions

The Sekem initiative from the ancient greek hieroglyph meaning "vitality from the sun" was founded 1977 by Dr. Ibrahim Abouleish. Under the vision of "sustainable human development", an untouched part of the Egyptian desert 60 km northeast of Cairo was converted into fertile land. The underlying idea is the pursuit of biodynamic agriculture and giving back to the community (http://www.sekem.com).

"In the midst of sand and desert I see myself standing before a well drawing water. Carefully I plant trees, herbs and flowers and wet their roots with the precious drops. The cool well water attracts human beings and animals to refresh and quicken themselves. Trees give shade, the land turns green, fragrant flowers bloom […].
For me this idea of an oasis in the middle of a hostile environment is like an image of the resurrection at dawn, after a long journey through the nightly desert. I saw it in front of me like a model before the actual work in the desert started. And yet in reality I desired even more: I wanted the whole world to develop."

(Dr. Ibrahim Abouleish)

Plant growth promotion – selected strains with antagonistic activity against plant- and opportunistic human pathogens

Yield losses caused by soil-borne pathogens is an increasing ecological problem not only in conventional, but also in the organic desert farming in Sekem (Egypt), where the plant material analysed in this study was sampled. However, when comparing cultivated farmland soil and uncultivated desert soil, the antagonistic potential nearly doubled after long-term organic agriculture demonstrating bacterial shifts when desert land is being cultivated. Microbial communities examined by Köberl *et al*. (2011) showed that organically farmed soil samples exhibited a higher diversity along with a higher amount of *Firmicutes* represented by *Bacillus* and *Paenibacillus* compared to the uncultured soil of the enclosed desert land. In comparison, isolates of antagonistic *Streptomyces* however were only found in the native desert soil. The genera mentioned are no "strangers" in the biocontrol society as all three have been verified regarding their biocontrol properties and some strains are already used as sustainable pesticides. Beside this microhabitat specificity between agriculture land and desert soil, a correlation of microbial communities specific for each plant examined (*Matricaria chamomilla* L., *Calendula officinalis* L., and *Solanum distichum* Schumach. and Thonn.) was detected (Köberl *et al*., 2011, 2013a, 2016). The most promising candidates out of all bacterial isolates regarding their *in vitro* antifungal (*Verticillium dahliae*, *Rhizoctonia solani*, and *Fusarium culmorum*), antibacterial (*Ralstonia solanacearum, Escherichia coli*) and antinematodal (*Meloidogyne incognita*) potential were *Streptomyces* sp. Wb2n-11 (formerly referred to as *Streptomyces subrutilus* Wb2n-11) isolated from desert soil in Sinai, *Bacillus amyloliquefaciens* Co1-6 (formerly referred to as *Bacillus subtilis* Co1-6) obtained from the rhizosphere of *Calendula officinalis* L., and *Paenibacillus polymyxa* Mc5Re-14 isolated from the endorhiza of *Matricaria chamomilla* L. Beside their plant growth promoting properties in an *ad planta* evaluation under desert farm conditions, the strains are characterized by a high salt tolerance as well as resistance against drought (Köberl *et al.*, 2014). In primed seedlings (*Bacillus amyloliquefaciens* Co1-6 and *Paenibacillus polymyxa* Mc5Re-14) of chamomile plants grown under field conditions even an enhancement of the production of flavonoids (apigenin-7-*O*-glucoside and apigenin) was verified (Köberl *et al*., 2013b; Schmidt *et al*., 2014). The three valuated strains showed a promising potential in the suppression of plant pathogens and the promotion of plant health and are auspicious candidates supporting sustainable agriculture even under arid desert soil conditions.

Paenibacillus polymyxa Mc5Re-14

Paenibacillus polymyxa strain Mc5Re-14 was isolated in April 2010 from the endorhiza of the German chamomile, *Matricaria chamomilla* L., cultivated on the organically managed Sekem farms in the north-eastern desert region of Egypt (30°22'88"N; 31°39'41"E). Mc5Re-14 exhibited broad-spectrum antagonism against soil-borne phytopathogenic fungi (*Verticillium dahliae*, *Fusarium culmorum*, *Rhizoctonia solani*) and nematodes (*Meloidogyne incognita*), and was also active against the opportunistic human pathogen *Escherichia coli.* Treatment of chamomile plants with Mc5Re-14 under field conditions resulted in elevated flavonoid contents of the blossoms (Köberl *et al*., 2015a).

Bacillus amyloliquefaciens Co1-6

Bacillus amyloliquefaciens Co1-6 was isolated in October 2009 from the rhizosphere of the pot marigold, *Calendula officinalis* L., cultivated on the organically managed Sekem farms in the north-eastern desert region of Egypt (30°22'88"N; 31°39'41"E). Co1-6 was selected as broad-spectrum antagonist exhibiting antifungal (*Verticillium dahliae*, *Rhizoctonia solani*, *Fusarium culmorum*), antibacterial (*Ralstonia solanacearum*), and nematicidal (*Meloidogyne incognita*) activity against soil-borne phytopathogens. For strains of the same population, induced systemic resistance of the host plant was identified as the major reason for their nematicidal activity. Treatment of chamomile plants (*Matricaria chamomilla* L.) with Co1-6 under field conditions resulted in elevated flavonoid contents of the blossoms (Köberl *et al*., 2015b).

Streptomyces sp. Wb2n-11

Streptomyces sp. strain Wb2n-11 was isolated from native desert soil collected in the Sinai desert in Egypt (30°35'01"N; 32°25'49"E) in October 2009, at a depth of 10-30 cm. Wb2n-11 was selected as broad-spectrum antagonist exhibiting antifungal (*Verticillium dahliae*, *Rhizoctonia solani*, *Fusarium culmorum*), antibacterial (*Ralstonia solanacearum*), and nematicidal (*Meloidogyne incognita*) activity against soil-borne phytopathogens (Köberl *et al*., 2015c).

Materials and Methods

Cultivation and characterization of lactobacilli

Finely chopped leaves of cabbage, *M. chamomilla* L. and *C. officinalis* L. were put in glass jars and mixed with 2% (w/w) NaCl. The approaches were mashed and weighted down to be pressed tightly and covered with liquid (0.85% NaCl solution) ensuring anaerobic conditions (Figure 1). Approaches were incubated at room temperature for six weeks. Samples were taken approximately every week by withdrawing 15 ml of the liquid and 5 g of the fermented plant material. Samples were ground with a mortar (Figure 2) and 3 x 2 ml were centrifuged (13 500 rpm, 20 min/4°C) per sample. The supernatant was discarded and the pellet was frozen at -70°C until the later DNA extraction using the FastDNA® SPIN Kit for Soil. After six weeks of fermentation, *Lactobacillales* were cultivated on MRS and R2A agar containing Cycloheximide [20 µg/ml] (preventing fungal growth) at 30°C under four different cultivation conditions, always plating out 100 µl of the ground fermentation broth with sterile glass beads in dilutions ranging from 10^{-2} up to 10^{-6}, each in duplicates. The first approach was incubated aerobically on R2A agar to obtain a broad spectrum of all cultivable bacteria, further approaches were performed on MRS agar, which enables luxuriant growth of LAB especially of fastidious, slower-growing types; incubations were done aerobically, anaerobically in a desiccator with AnaeroGen packs (Oxoid, Basingstoke, UK), and microaerobically performing the pour plating method. Single colonies based on their morphological differences were picked from each of the four cultivation methods and from each of the seven different plant approaches:

Figure 1: Leaves of *Calendula officinalis* L. in a glass jar during fermentation.

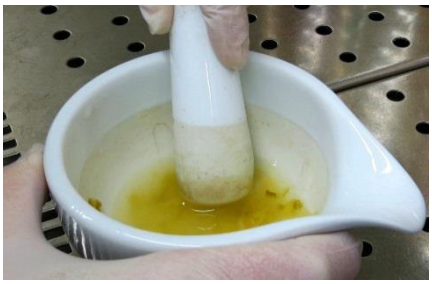

Figure 2: Fermented leaves of cabbage beeing pestled in the course of the cultivation of lactic acid bacteria.

- leaves of *M. chamomilla* L. (Sekem farm),
- leaves of *M. chamomilla* L. (Faiyum oasis),
- leaves of *C. officinalis* L. (Sekem farm),
- leaves of *C. officinalis* L. (Faiyum oasis),
- "big" cabbage (Egypt),
- "small" cabbage (Egypt),
- cabbage (Austria)

CFUs were calculated of all different approaches and calculated using Equation 1.

Equation 1: Formula for the calculation of colony forming units (CFU) per milliliter.

$$\frac{cfu}{ml} = \frac{number\ of\ colonies\ x\ dilution}{plated\ volume\ [ml]}$$

Six colonies were streaked out per plate whereas colonies grown on R2A were again streaked on R2A, while colonies grown on MRS beforehand were grown on MRS. Incubation of the isolates was done at 30°C. Colonies picked from the anaerobic approaches and from the pour plating method were incubated anaerobically, colonies picked from the other two approaches were incubated aerobically. Grown isolates were subjected to a KOH test (cell material was mixed with 3% KOH-solution) as well as a Katalase test (cell material was mixed with 3% H_2O_2-solution) and only colonies which could be classified as potential lactic acid bacteria (Gram-positive in the KOH test and Katalase-negative) –394 isolates, were used for a dilution streak by transferring two isolates per plate. All plates were incubated aerobically. To assure having only one strain per sample and no contamination for the later DNA isolation and the preparation of conserves, another dilution streak was done by picking one single colony of the first dilution streak and streaking it out on another plate. Again, all the plates were incubated aerobically at 30°C. After incubation, cell material from single colonies was picked for preserving the isolates in 600 µl NBII and 400 µl 40% glycerol. For the DNA preparation, cell material of each of the 400 isolates was transferred in reaction tubes with screw stoppers each filled with 200 µl nuclease free water and glass beads. Samples were undertaken a mechanical cell disruption step using a ribolyser (30 sec/6.5 ms^{-1}) before treatening the samples with the heating method (frozen at -20°C, heated for 5 min. at 100°C and centrifuged 5 min. at 4°C and 135000 rpm).

In vitro antagonistic potential of potential lactic acid bacteria

A dual culture assay was performed to test the antagonistic potential of the ~400 isolates of the fermentation approaches against different plant-pathogenic fungi and against potential human pathogens. Thereby, antagonistic tests against the fungi *Fusarium culmorum* E1, *Verticillium dahliae* V25 and *Rhizoctonia solani* AG4 were done on Waksman agar. Antagonistic tests against the human pathogens *E. coli* K12, *Salmonella typhimurium* and *Staphylococcus aureus* were performed on TSB-Agar. As positive controls, the BioGuard strains *Bacillus amyloliquefaciens* Co1-6, *Paenibacillus polymyxa* Mc5Re-14, *Streptomyces*

sp. Wb2n-11 and *Bacillus subtilis* Mc5Re-2 which showed antagonistic effects in previous studies (Köberl *et al.*, 2013a) were tested. Always four potential antagonists were applied per plate with sterile toothpicks. For the approaches with *Verticillium dahliae* V25 0.2 ml of inoculated Czapek Dox Broth (Duchefa Biochemie; Haarlem, Netherlands) were plated out on the Waksman agar before applying the potential antagonists per plate. To test the antagonistic potential against *Rhizoctonia solani* AG4, one, with a cork borer, punched-out piece of fungal-infected agar was put in the middle of the plate, for *Fusarium culmorum* E1 four punched-out pieces of fungal-infected agar were applied. An ONC (30°C, 220 rpm) of each of the human pathogens was prepared and 2 x 50 ml TSB media (A & B) were inoculated with the ONCs to reach an OD_{600} of 0.2. After 60 min. and 105 min. the optical density was measured and had ranged the desired OD_{600} between 0.6-0.8 (Table 1). Afterwards, 50 ml of the prepared cultures were added to 450 ml (moderate temperature) TSB-agar and plates were poured before the antagonists were applied. All antagonistic test plates were incubated at room temperature and the growth was examined every day.

Table 1: Meassured optical density (600 nm) of inoculated TSB medium with human pathogens after different points of times (60 min/105 minutes).

Pathogen	After 60 min. incubation		After 105 min. incubation	
	A	B	A	B
Staphylococcus aureus	0.320	0.324	0.548	0.641
***E. coli* K12**	0.488	0.472	0.806	0.829
Salmonella typhimurium	0.443	0.443	0.721	0.738

Amplified ribosomal RNA gene restriction analysis (ARDRA)

To select and group the isolates of the *Lactobacillus* cultivation, the method of Amplified Ribosomal DNA Restriction Analysis (ARDRA) was applied. The 16S rRNA gene of all isolates was amplified in a PCR using the universal primer pair 27f/1492r (Lane *et al.*, 1991) in a total reaction mix of 20 µl per sample (4 µl Taq&Go, 1 µl of each primer [10 pmol/µl], 1 µl DNA, 13 µl H_2O). After amplification (95°C 5 min, 30 cycles of 95°C 20 sec, 54°C 15 sec. 72°C 30 sec and 72°C 10 min) 5 µl of the PCR product were applied to an agarose control gel (0.8% Agarose, 1 x TAE) before performing a restriction digest with the enzyme *Hha*I for 3 h at 37°C in a total volume of 20 µl consisting of 15 µl DNA, 0.5 µl *Hha*I [10U/µl], 2 µl buffer NEB4 (New England Biolabs; Ipswich, USA), 0.2 µl BSA [100mg/ml] and 2.3 µl UVclear H_2O. Samples were analyzed through gel electrophoresis by applying the whole digestion approach (20 µl together with 6 x LD) to a 2% agarose gel. Electrophoresis was done in 0.5 x TBE at 120V for 4 h. Isolates were divided into groups (A-E) according to their different

digestion patterns. From each group, random samples were selected and subjected to 16S rRNA gene sequencing (LGC Genomics; Berlin, Germany) to check their genetic affiliation to the lactic acid bacteria. Results of the sequencing were aligned with the NCBI BLAST algorithm against the reference RNA sequence database.

rep-PCR fingerprinting (BOX-PCR)

BOX-PCR was done to further differentiate the isolates. Therefore, a PCR was implemented using the BOX_A1 primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') as described by Rademaker & de Bruijn (1997). Amplification in a thermocycler (Biometra; Goettingen, Germany) was carried out in a total volume of 15 µl consisting of 9.5 µl nuclease-free, steam sterilized, DEPC treated H_2O (Roth; Karlsruhe, Germany), 1.5 µl primer BOX_A1 [10 pmol μl^{-1}], 3 µl 5 x Taq&Go™ Ready-to-use PCR Mix (MP Biomedicals; Santa Ana, USA) and 1 µl template DNA (95°C 6 min; 35 cycles of 94°C 1 min, 53°C 1 min, 65°C 8 min; and 65°C 16 min). The BOX-PCR approaches were analyzed by gel-electrophoresis with a 1.5% agarose gel. The peqGOLD universal agarose (Peqlab; Erlangen, Germany) was mixed with 0.5 × TBE buffer and heated in the microwave until the solution turned transparent and streak-free. The mix was put at 60°C and the gel was poured the following day. The hardened gel was covered with 0.5 × TBE buffer and the whole PCR approach (15 µl each) mixed with 3 µl 6 × loading dye (Thermo Scientific, Waltham, USA) was applied to the gel. As a standard, 5 µl of the GeneRuler™ 1 kb DNA Ladder Ready-to-Use (Thermo Scientific, Waltham, USA) were added. The gel ran for 4 h (120 V, 300 mA, 50 W). Resulting bands were visualized under an UV transilluminator (Gel Doc 2000; Bio-Rad; Hercules, USA) after the staining with ethidium bromide.

16S rRNA gene amplicon sequencing

To further and deeper characterize the colonization profiles of medicinal plants, amplicon sequencing of 16S rRNA genes was implemented on the one hand of the fresh samples of the medicinal plants from the phyllospheric endo- and ectosphere and on the other hand of the fermented plant samples covering a weekly fermentation period of six weeks. To block the hosts chloroplastic and mitochondrial DNA contaminations, a PCR using the PNA-clamps pPNA and mPNA was performed (Lundberg *et al.*, 2013). Per sample, a 30 µl approach was

done consisting of 6.0 µl Taq & Go (5x), 1.2 µl barcoded primer 515f_BC [5.0 µM], 1.2 µl barcoded primer 806r_BC [5.0 µM] (Caporaso *et al.*, 2011), 0.225 µl of each of the PNAs [100 µM], 19.15 µl water and 2.0 µl DNA (~1.5 ng pro 30 µl). Amplification program started with a denaturation step at 96°C for five minutes followed by 30 cycles of 96°C, 1 min., 78°C, 5 sec. (PNA annealing), 54°C, 1 min. (primer annealing), 74°C, 1 min. and elongation at 74°C for 10 min. PCR products were subjected to an electrophoresis (0.8% agarose, 1 x TAE), and separated bands were visualized under the UV transilluminator to check the amplification of the right product. PCR of each sample was done in triplicates, which were pooled together (3 x 30 µl) in the cleaning step using the Wizard® SV Gel and PCR Clean-Up System (Promega; Fitchburg, USA). After eluting the DNA in 30 µl nuclease free water the concentration was measured using a Nanodrop spectrophotometer. All approaches were then pooled together in an equimolar ratio and subjected to Illumina MiSeq sequencing (chemistry v3, 250 bp paired-end) at GATC Biotech (Konstanz, Germany). Raw sequencing paired-end reads were assembled with default settings of PANDAseq software, version 2.8 (Masella *et al.*, 2010). Barcode and primer sequences were trimmed by PRINSEQ software, version 0.20.4 (Schmieder & Edwards, 2011). In addition, low-quality reads defined as reads with an average quality score below 25, with more than one ambiguous base and a length <250 and >260 were removed using PRINSEQ. The processing of filtered reads to operational taxonomic units (OTUs) was done with the LotuS program using UPARSE at 97% similarity and USEARCH in subsequent seed extension (Hildebrand *et al.*, 2014). OTUs assigned to plant-derived chloroplasts, mitochondria and archaeal 16S rRNA were filtered from the dataset by QIIME 1.9.1 (Caporaso *et al.*, 2010), which was also used for further microbiome and statistical analyses. For alpha and beta diversity analyses, OTU tables were normalized to the same number of quality reads per sample. Statistical analyses for comparing alpha diversity measurements were performed using the nonparametric *t*-test with 999 Monte Carlo permutations. Beta diversity was analyzed based on Bray-Curtis distances; the adonis test with 999 permutations was used for corresponding statistics. Significant differences at OTU level were ascertained with Metastats (White *et al.*, 2009), where p values were computed using a combination of the nonparametric *t*-test, exact Fisher's test, and the false discovery rate with 10^3 permutations.

Single strand conformational polymorphism (SSCP) analysis

Cultivation-independent PCR-single-strand conformation polymorphism (SSCP) analysis was done to get an overview of the colonization of the phyllosphere of different medicinal plants grown on Sekem farms. DNA of conserved (year 2012) phyllosphere samples from at least 5 different plants of *M. chamomilla* L., *C. officinalis* L. and *S. distichum* Schumach. and Thonn. was extracted and SSCP-PCRs were done, capturing different taxonomic groups of bacteria and fungi. In addition to the medicinal plants, samples of fresh *Brassica oleracea* L. var. *capitata* (cabbage "small" & cabbage "big" from Sekem farms as well as cabbage from Austria) were subjected to SSCP analysis. SSCP was also implemented for the fermentation approaches covering the *Lactobacilli* of the weekly withdrawn samples of the two medicinal plants. DNA of the different plant samples was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals; Santa Ana, USA). The extraction was done according to the manufacturers' protocol. Without any further purification of the DNA after the extraction, various PCRs were performed, capturing taxonomically different bacteria and fungi by applying varying primers (Table 2). The reverse primer used for all PCRs was phosphorylated at the end to provide a starting point for the later Lambda exonuclease digestion. After amplification, control gels of all the PCR approaches (0.8% agarose, 1 x TAE, 5 µl of the PCR approach + 2 µl 6 x LD, 110 V, 30 min) were implemented.

Table 2: Primers with varying specificity used for the SSCP analysis of various bacterial communities isolated from different plant species.

Primer name	Primer sequence (5'-3')	Specificity	Reference
Unibac-II-515f	GTG CCA GCA GCC GC	*Eubacteria*	Lieber *et al.*, 2003
Unibac-II-927r^{P}	CCC GTC AAT TYM TTT GAG TT	*Eubacteria*	Lieber *et al.*, 2003
Actino243f	GGA TGA GCC CGC GGC CTA	*Actino-mycetales*	Heuer *et al.*, 1997
Actino513r^{P}	CGG CCG CGG CTG GTG GCA CGT A	*Actino-mycetales*	Heuer *et al.*, 1997
BLS342F	CAG CAG TAG GGA ATC TTC	*Bacilli*	Blackwood *et al.*, 2005
BACr833^{P}	CTA ACA CTT AGC ACT CAT	*Firmicutes*	Fu *et al.*, 2011
27f	AGA GTT TGA TCC TGG CTC AG	*Eubacteria*	Lane *et al.*, 1991
1492r	TAC GGY TAC CTT GTT ACG ACT T	*Eubacteria*	Lane *et al.*, 1991
F311Ps	CTG GTC TGA GAG GAT GAT CAG T	Pseudomonads	Milling *et al.*, 2004
R1459Ps	AAT CAC TCC GTG GTA AAC GT	Pseudomonads	Milling *et al.*, 2004

Gamma395f	CMA TGC CGC GTG TGT GAA	*Gamma-proteobacteria*	Mühling *et al.*, 2008
Gamma871r^{P}	ACT CCC CAG GCG GTC DAC TTA	*Gamma-proteobacteria*	Mühling *et al.*, 2008
LactF	AGC AGT AGG GAA TCT TCC A	*Lactobacillus*	Bell *et al.*, 2014
LactR^{P}	CAC CGC TAC ACA TGG AG	*Lactobacillus*	Bell *et al.*, 2014
ITS1F	TCC GTA GGT GAA CCT GCG G	Fungi	White *et al.*, 1990
ITS4A	TCC TCC GCT TAT TGA TAT GC	Fungi	White *et al.*, 1990
ITS1	TCC GTA GGT GAA CCT GCG G	Fungi	White *et al.*, 1990
ITS2^{P}	TCC TCC GCT TAT TGA TAT GC	Fungi	White *et al.*, 1990

Subsequent, the various conducted PCRs with optimized amplification conditions and using distinct primer pairs for each bacterial community colonizing the investigated plants are listed. All amplifications were performed in a thermocycler (Biometra; Goettingen, Germany) and additives for the amplifications were as follows: nuclease-free, steam sterilized, DEPC treated H_2O (Roth; Karlsruhe, Germany), Taq&Go^{TM} Ready-to-use PCR Mix (MP Biomedicals; Santa Ana, USA), and 25 mM $MgCl_2$ (New England Biolabs; Ipswich, USA).

Total bacteria community

To cover the total bacteria community, a PCR approach using the universal eubacterial primers Unibac-II-515f and Unibac-II-927r^{P} were used. Amplification was carried out in a total volume of 60 µl per approach consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol µl^{-1}] and 6 µl template DNA (95°C 5 min.; 32 cycles of 95°C 20 s., 54°C 15 s., 72°C 30 s.; and 72°C 10 min.).

Actinomycetales cummunity

The *Actinomycetales* community was analyzed by performing a PCR carried out using the primer pair Actino243f and Actino513r^{P} in a total volume of 60 µl per approach consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol µl^{-1}] and 6 µl template DNA (94°C 5 min.; 35 cycles of 94°C 1 min., 63°C 1 min., 72°C 2 min.; and 72°C 10 min.).

Firmicutes community

A PCR with the primers BLS342F und BACr833^{P} was implemented in a total volume of 60 µl consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 6 µl template DNA (95°C 5 min.; 28 cycles of 95°C 45 s., 54°C 60 s., 72°C 45 s.; and 72°C 10 min.). In the course of the experiment though it was noticed, that the BLS342F und BACr833^{P} primers did not cover the genus *Lactobacillus*, therefore, additional PCRs specifically designed for amplification of the *Lactobacillus* community were implemented. A nested PCR approach was conducted, where the first PCR was carried out in 20 µl total reaction volume per approach containing 4 µl 5 x Taq&Go Ready-to-use PCR Mix, 1.2 µl 25 mM $MgCl_2$, 0.8 µl Primer (5 pmol/µl), 12.2 µl H_2O and 1 µl template DNA using the primer pair BLS342F and 1492R (95°C 5 min.; 30 cycles of 95°C 30 s., 57°C 30 s., 72°C 70 s.; and 72°C 10 min.). The second PCR was implemented with the universal primers Unibac-II-515f and Unibac-II-927r^{P} (95°C 5 min.; 32 cycles of 95°C 20 s., 54°C 15 s., 72°C 30 s.; and 72°C 10 min.) in a total reaction volume of 55 µl consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 1 µl template DNA (from the first PCR approach (undiluted).

Gammaproteobacteria community

The PCR was done using the primers Gamma395f and Gamma871r^{P} in a total volume of 60 µl per approach consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 6 µl template DNA (96°C 4 min.; 30 cycles of 96°C 1 min., 54°C 1 min., 74°C 1 min.; and 74°C 10 min.).

Lactobacillus community

The first PCR was carried out in 20 µl total reaction volume per approach containing 4 µl 5 x Taq&Go Ready-to-use PCR Mix, 1.2 µl 25 mM $MgCl_2$, 0.8 µl Primer (5 pmol/µl), 12.2 µl H_2O and 1 µl template DNA using the universal primer pair 27f and 1492r (95°C 5 min.; 30 cycles of 95°C 30 s., 57°C 30 s., 72°C 1 min. 30 s.; and 72°C 5 min.). The second PCR was implemented with the specific primers for the *Lactobacillus* community LactF and LactR^{P} (95°C 5 min.; 32 cycles of 95°C 20 s., 58°C 15 s., 72°C 30 s.; and 72°C 10 min.) in a total reaction volume of 60 µl per approach consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 6 µl template DNA from the first PCR approach (1:100 dilution).

Pseudomonas community

The first PCR was carried out in 20 µl total reaction volume per approach containing 4 µl 5 x Taq&Go Ready-to-use PCR Mix, 1.2 µl 25 mM $MgCl_2$, 0.8 µl Primer (5 pmol/µl), 12.2 µl H_2O and 1 µl template DNA using the primer pair F311Ps and R1459Ps (94°C 7 min.; 30 cycles of 94°C 45 s., 56°C 2 min., 72°C 2 min.; and 72°C 10 min.). The second PCR was implemented with the universal primer pair Unibac-II-515f and Unibac-II-927r^P. Amplification was carried out in a total volume of 60 µl consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 6 µl template DNA from the first PCR approach (1:1000 dilution) at 95°C 5 min.; 32 cycles of 95°C 20 s., 54°C 15 s., 72°C 30 s.; and 72°C 10 min.

Total fungal community

The first PCR was carried out in 20 µl total reaction volume per approach containing 4 µl 5 x Taq&Go Ready-to-use PCR Mix, 1.2 µl 25 mM $MgCl_2$, 0.8 µl Primer (5 pmol/µl), 12.2 µl H_2O and 1 µl template DNA using the primer pair ITS1 and ITS4 (95°C 5 min.; 37 cycles of 95°C 30 s., 54°C 35 s., 72°C 40 s.; and 72°C 10 min.). The second PCR was implemented with the primer pair ITS1 and ITS2^P. Amplification was carried out in a total volume of 60 µl consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 6 µl template DNA from the first PCR approach (1:1000 dilution) at 95°C 5 min.; 37 cycles of 95°C 30 s., 58°C 35 s., 72°C 40 s.; and 72°C 10 min.

Ascomycota community

The first PCR was carried out in 20 µl total reaction volume per approach containing 4 µl 5 x Taq&Go Ready-to-use PCR Mix, 1.2 µl 25 mM $MgCl_2$, 0.8 µl Primer (5 pmol/µl), 12.2 µl H_2O and 1 µl template DNA using the primer pair ITS1F and ITS4A (95°C 5 min.; 37 cycles of 95°C 30 s., 54°C 35 s., 72°C 40 s.; and 72°C 10 min.). The second PCR was implemented with the primer pair ITS1 and ITS2^P. Amplification was carried out in a total volume of 60 µl consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 6 µl template DNA from the first PCR approach (1:1000 dilution) at 95°C 5 min.; 37 cycles of 95°C 30 s., 58°C 35 s., 72°C 40 s.; and 72°C 10 min.

As in the amplicon sequencing, attempts were undertaken to differentiate colonization of the ecto- and endosphere of the medicinal plants using SSCP. Therefore, PCRs using different

primers to capture diverse bacterial communities were implemented. Among them, primers for the amplification of the total bacteria community (Unibac-II-515f/Unibac-II-927r^P), *Actinomycetales* (Actino243f/Actino513r^P), *Firmicutes* (BLS342F/1492R and Unibac-II-515f/Unibac-II-927r^P), *Gammaproteobacteria* (Gamma395f/Gamma871r^P) and partially nested PCR approaches using primers capturing the total fungi community (ITS1F/ITS4), *Ascomycota* (ITS1F/ITS4A) and pseudomonads (F311Ps/R1459Ps) were used. Regarding the weekly withdrawn fermentation approaches of the two medicinal plants (*M. chamomilla* L. and *C. officinalis* L.) a nested PCR for the analysis of the *Lactobacillus* community of the fermented plant samples was performed. The first PCR was carried out in 20 µl total reaction volume per approach containing 4 µl 5 x Taq&Go Ready-to-use PCR Mix, 1.2 µl 25 mM $MgCl_2$, 0.8 µl Primer (5 pmol/µl), 12.2 µl H_2O and 1 µl template DNA using the universal primer pair 27f and 1492r (95°C 5 min.; 30 cycles of 95°C 30 s., 57°C 30 s., 72°C 1 min. 30 s.; and 72°C 5 min.). The second PCR was implemented with the specific primers for the *Lactobacillus* community LactF and LactR^P (95°C 5 min.; 32 cycles of 95°C 20 s., 58°C 15 s., 72°C 30 s.; and 72°C 10 min.) in a total reaction volume of 60 µl per approach consisting of 33.6 µl H_2O, 12 µl 5 x Taq&Go^{TM} Ready-to-use PCR Mix, 3.6 µl $MgCl_2$, 2.4 µl of each primer [5 pmol μl^{-1}] and 6 µl template DNA from the first PCR approach (1:100 dilution). After PCR amplification, the PCR approaches were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega; Fitchburg, USA) following the manufacturer's protocol. The DNA was eluted from the minicolumn with 31 µl nuclease free water and subjected to Lambda exonuclease digestion (37°C, 60 min) using 2.4 µl Lambda exonuclease (New England Biolabs*;* Ipswich, USA) and 3.6 µl 10x Lambda exonuclease buffer (New England Biolabs; Ipswich, USA) per sample. 30 µl loading dye (950 µl formamide, 4 µl NaOH [10 mM], 5 µl bromophenol blue (0,025%) and 41 µl nanopure H_2O) were added to the nuclease digested samples and put at 95°C for 5 min. for denaturation followed by the renaturation on ice for minimum 5 min. to allow single strand folding. The polyacrylamide gel solution (Table 3) was prepared and transferred to the adjusted SSCP chamber (equipped with the support film).

Table 3: Composition of polyacrylamide gel solutions with varying degree of polymerization (8%/9%) for SSCP analysis of various bacterial communities isolated from different microenvironments.

Concentration	8%	9%
2 x MDE solution	18.2 ml	20.5 ml
5 x TBE	10.4 ml	10.4 ml
TEMED	24.5 µl	27.5 µl
10% APS	245 µl	275 µl
Nanopure H_2O	23.1 ml	20.8 ml

After polymerization, the gel was transferred to the Teflon plate of the TGGE Maxi System (Biometra; Goettingen, Germany), buffer chambers were filled with 1x TBE buffer and cellulose membranes were put in each buffer chamber and applied on each side of the gel to allow current flow. A protective sheet was put on the gel before starting the forerun (400 V, 50 mV, 20°C, 10 min.). Then, 12 µl per sample were applied per slot, flanked by two slots of 2 µl GeneRuler™ 1 kb DNA Ladder Ready-to-Use mix on both sides of the gel and the electrophoresis was performed at 400 V, 50 mA, 26°C. Concentration of the polyacrylamide gel and duration of the electrophoresis was dependent on the sample and can be gathered from Table 4.

Table 4 : Varying degree of polymerization and electrophoresis time of polyacrylamide gels deployed for SSCP analysis of various bacterial communities isolated from different microenvironments.

PCR sample approach	Polyacrylamide gel [%]	Electrophoresis time [h]
Total bacterial community	8	26
Firmicutes community	8	26
Pseudomonas community	8	26
Gammaproteobacteria community	8	26
Total fungi community	9	17
Ascomycota community	9	17
Lactobacillus community	9	17
Actinomycota community	9	17

SSCP gels were subjected to silver staining based on the method of Bassam *et al.* (1991). Fixation was done with 10% acetic acid for 30 min, followed by a washing step (3 times, 5 minutes) with distilled water. Staining was performed using a 0.1% silver nitrate solution with 5 ml 37% HCOH/liter. After washing for 10 sec. with distilled water, gels were developed under exclusion of light with a 3% NaOH solution supplied with 4 ml 37% HCOH/liter until bands appeared (approximately 5 min). To stop the reaction, 10% acetic acid was used for 30 minutes. The gels were conserved in a conserving solution containing 10% ethanol and 13% glycerol and visualized bands were undertaken analysis using the software program GelCompar II (Version 5.1; Applied Maths; Sint-Martens-Latem, Belgium). For later 16S rRNA sequencing, dominant bands were excised from the gels with a flamed scalpel and frozen for 1 h at -20°C in 150 µl Crush and Soak buffer according to Schwieger & Tebbe (1998). Samples were put in the heating block at 37°C for 3 h and incubated at 4°C for 3-5 days. Centrifugation was carried out at 13 000 rpm for 20 min. and 4°C, supernatant was transferred into a sterile 1.5 ml reaction tube and DNA was precipitated over night at -20°C with 150 µl isopropanol. Samples were subjected to a 20 min. centrifugation step at 4°C and 13 000 rpm, supernatant was discarded and the pellets were washed twice, performing 10 min. centrifugations at 4°C and 13 000 rpm and washing steps with 70% ethanol. Ethanolic

supernatant was discarded and the pellet resuspended in 50 µl 10 mM Tris-HCl after drying under the clean bench. Gathered DNA was reamplified performing PCRs with the same primer pair used for the previous SSCP analysis in a total volume of 30 µl per approach (21 µl DNA, 6 µl Taq&Go, 1.8 µl $MgCl_2$ [25 mM], 1.2 µl of each primer [5 pmol μl^{-1}]). Control gels were done with 0.8% agarose gels and 1x TAE puffer. PCR approaches were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega; Fitchburg, USA) following the manufacturer's protocol. Reamplified DNA was send to LGC Genomics (Berlin, Germany) for sequencing. Sequencing results were subjected to a "megablast" similarity search (highly similar sequences) using the nucleotide BLASTn alignment tool of the National Center for Biotechnology Information (NCBI) against the reference sequence RNA (refseq_RNA) database.

Fluorescence *in situ* hybridization and confocal laser scanning microscopy

Fresh cabbage, leaves of *M. chamomilla* L. and *C. officinalis* L. as well as fermented plant samples (after 5 weeks of fermentation) of cabbage, *M. chamomilla* L. and *C. officinalis* L. were analyzed for their colonization with different bacteria using confocal laser scanning microscopy (CLSM). Samples therefore were either paraformaldehyde or ethanol fixated. The fixation protocol and solution preparation therefore can be gathered from the appendix. In addition to the aboveground samples also conserved (4% paraformaldehyde-fixated) and frozen roots of *M. chamomilla* L. (harvested from Adleja Farm/Sekem in year 2012) were analyzed. Plant material was cut into small pieces with a razor blade and hybridized with different FISH probes to visualize colonization patterns of bacteria on selected plant samples. Aerial plant material (medicinal plants and cabbage) was prepared to examine the colonization patterns of Gram-negative *Gammaproteobacteria* and Gram-positive *Firmicutes* covering lactic acid bacteria. Following the protocol of Cardinale *et al*. (2008), samples were first hybridized with different FISH probes [LGC354A, LGC354B and LGC354C (Meier *et al.*, 1999) with the fluorochrome FITC; GAM42a (Manz *et al.*, 1992) with the fluorochrome Cy5 in combination with the unlabeled GAM42a-competitor at 45% formamide concentration and EUB338, EUB338II and EUB338III with the fluorochrome Cy3 (Amann *et al.*, 1990; Daims *et al.*, 1999)] at a 10% formamide concentration (Table 5). The rhizosphere of conserved *M. chamomilla* L. was analyzed for the colonization with Gram-negative *Rhizobiales*. Following the protocol of Cardinale *et al*. (2008), the first hybridization step was

done using the FISH probes RHIZ1244-Cy5 (Thayanukul *et al.*, 2010) and 3R-FISH-Cy5 (Nishio *et al.*, 1997) with 45% formamide concentration. The second hybridization was performed using the EUB-mix-Cy3 (Amann *et al.*, 1990; Daims *et al.*, 1999) at 10% formamide concentration (Table 5). Negative controls were prepared by hybridization with the NON-EUB-mix with either the Cy5 or Cy3 fluorochrome (Wallner *et al.*, 1993) FISH probe (Table 5). Parameters for all implemented the hybridizations were set as follows: hybridization puffer heated at 43°C; washing puffer heated at 44°C; hybridization time per hybridization step: 90 min. Samples were put into the refrigerator before being analyzed with a Leica TCS SPE microscope (Leica Microsystems; Wetzlar, Germany).

Table 5: FISH probes applied for the investigation using FISH-CLSM of fermented and unfermented plant samples for their colonization with different bacterial communities.

Name	Sequence (5'- -3')	Fluorescent	Target	FA (%)	Reference	
Rhizobiales						
RHIZ1244	TCGCTGCCCA GTCACC	Cy5	*Rhizo-biales*	45	Thayanukul *et al.*, 2010	1. hybridisation
3R-FISH	GGCTTATCAC GTCTCC	Cy5	Metylo-trophs/ *Brady-rhizobium*	45	Nishio *et al.*, 1997	
EUB338	GCT GCC TCC CGT AGG AGT	Cy3	Most bacteria	10	Amann *et al.*, 1990	2. hybridisation
EUB338II	GCA GCC ACC CGT AGG TGT	Cy3	*Plancto-mycetales*	10	Daims *et al.*,1999	
EUB338III	GCT GCC ACC CGT AGG TGT	Cy3	*Verruco-microbiales*	10	Daims *et al.*,1999	
NONEUB-Cy5	ACT CCT ACG GGA GGC AGC	Cy5	-	45	Wallner *et al.*, 1993	negative controls
NONEUB-Cy3	ACT CCT ACG GGA GGC AGC	Cy3	-	10	Wallner *et al.*, 1993	
Firmicutes/ Gamma-proteobacteria						
LGC354A	TGG AAG ATT CCC TAC TGC	FITC	Part of *Firmicutes* (Low G+C Gram-positive bacteria)	45	Meier *et al.*, 1999	1. hybridisation
LGC354B	CGG AAG ATT CCC TAC TGC	FITC	Part of *Firmicutes* (Low G+C Gram-positive bacteria)	45	Meier *et al.*, 1999	

LGC354C	CCG AAG ATT CCC TAC TGC	FITC	Part of *Firmicutes* (Low G+C Gram-positive bacteria)	45	Meier *et al*., 1999	
GAM42a	GCC TTC CCA CAT CGT TT	Cy5	*Gamma-proteobacteria*	45	Manz *et al*., 1992	1. hybridisation
BET42a-competitor	GCC TTC CCA CAT CGT TT	-	*Gamma-proteobacteria*	45	Manz *et al*., 1992	
EUB338	GCT GCC TCC CGT AGG AGT	Cy3	Most bacteria	10	Amann *et al*., 1990	
EUB338II	GCA GCC ACC CGT AGG TGT	Cy3	*Plancto-mycetales*	10	Daims *et al*.,1999	2. hybridisation
EUB338III	GCT GCC ACC CGT AGG TGT	Cy3	*Verruco-microbiales*	10	Daims *et al*.,1999	
NONEUB-Cy3	ACT CCT ACG GGA GGC AGC	Cy3	-	10	Wallner *et al*., 1993	
NONEUB-Cy5	ACT CCT ACG GGA GGC AGC	Cy5	-	45	Wallner *et al*., 1993	negative controls
NONEUB-ATTO488	ACT CCT ACG GGA GGC AGC	ATTO488	-	45	Wallner *et al*., 1993	

Fingerprint analysis of antagonistic strains

To create fingerprints of 40 isolated strains (Table 6) tested for their antagonistic activity against different soil-borne pathogens (Köberl *et al*., 2013a), a BOX-PCR using the BOX_A1 primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') as described by Rademaker & de Bruijn (1997) was performed. Amplification in a thermocycler (Biometra; Goettingen, Germany) was carried out in a total volume of 15 µl consisting of 9.5 µl nuclease-free, steam sterilized, DEPC treated H_2O (Roth; Karlsruhe, Germany), 1.5 µl primer BOX_A1 [10 pmol μl^{-1}], 3 µl 5 x Taq&Go™ Ready-to-use PCR Mix (MP Biomedicals; Santa Ana, USA) and 1 µl template DNA (95°C 6 min; 35 cycles of 94°C 1 min, 53°C 1 min, 65°C 8 min; and 65°C 16 min). The BOX-PCR approaches were analyzed by gel-electrophoresis with a 1.5% agarose gel. The peqGOLD universal agarose (Peqlab; Erlangen, Germany) was mixed with 0.5 × TBE buffer and heated in the microwave until the solution turned transparent and streak-free. The mix was put at 60°C and the gel was poured the following day. The hardened gel was covered with 0.5 × TBE buffer and the whole PCR approach (15 µl each) mixed with 3 µl 6 × loading dye (Thermo Scientific, Waltham, USA) was applied to the gel. As a standard, 5 µl of the GeneRuler™ 1 kb DNA Ladder Ready-to-Use (Thermo Scientific,

Waltham, USA) were added. The gel ran for 4 h (120 V, 300 mA, 50 W). Resulting bands were visualized under an UV transilluminator (Gel Doc 2000; Bio-Rad; Hercules, USA) after the staining with ethidium bromide. Bands were clustered through the creation of a dendrogram using the software program GelCompar II (Version 5.1; Applied Maths; Sint-Martens-Latem, Belgium) under the application of the curve-based Pearson's correlation similarity coefficient. The dendrogram type unweighted paired group means algorithm (UPGMA) was chosen, position tolerance was set to optimization 4% and position tolerance adjusted to 1%.

Table 6: Selected strains tested for their antagonistic potential against soil-borne pathogens, grouped after their varying amplified rRNA gene restriction analysis patterns. (table content taken from Köberl *et al.*, 2013a).

Number	ARDRA group[+]	Isolate designation	Closest database match[*] (accession number), similarity (%)
1	A	Wb2n-1	*Bacillus vallismortis* (NR_024696), 99%
2	A	Sb1-6	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
3	A	Sb3-5	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
4	A	Sb3-13	*Bacillus atrophaeus* (NR_024689), 99%
5	A	Sb3-21	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%
6	A	Sb3-24	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
7	A	Sb4-14	*Bacillus vallismortis* (NR_024696),99%
8	A	Sb4-23	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
9	A	Mc3-4	*Bacillus mojavensis* (NR_024693), 98%
10	A	Mc5-18	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
11	A	Mc5-19	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
12	A	Co1-6	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
13	A	Co2-14	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%
14	A	Co7-19	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%
15	A	Sd1-14	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%
16	A	Sd3-12	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 100%
17	A	Sd3-21	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%
18	A	Sd7-15	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%

19	A	Mc1Re-3	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
20	A	Mc2Re-2	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%
21	A	Mc2Re-9	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
22	A	Mc2Re-18	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
23	A	Mc2Re-21	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
24	A	Mc3Re-13	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 98%
25	A	Mc5Re-2	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%
26	A	Mc5Re-15	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
27	A	Sd2Re-10	*Bacillus mojavensis* (NR_024693), 100%
28	A	Sd8Re-6	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%
29	A	Sd8Re-7	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%
30	A	Sd8Re-23	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%
31	C	Wb1-13	*Bacillus endophyticus* (NR_025122), 99%
32	C	Mc4-18	*Bacillus endophyticus* (NR_025122), 99%
33	D	Wb2-3	*Paenibacillus polymyxa* (NR_037006), 99%
34	D	Sb3-1	*Paenibacillus kribbensis* (NR_025169), 99%
35	D	Mc2-9	*Paenibacillus brasilensis* (NR_025106), 99%
36	D	Mc5-5	*Paenibacillus brasilensis* (NR_025106), 99%
37	D	Mc6-4	*Brevibacillus limnophilus* (NR_024822), 99%
38	D	Mc2Re-16	*Paenibacillus brasilensis* (NR_025106), 98%
39	D	Mc5Re-14	*Paenibacillus polymyxa* (NR_037006), 99%
40	D	Sd5Re-24	*Paenibacillus brasilensis* (NR_025106), 99%

[+] The letters represent the different amplified rRNA gene restriction analysis patterns (A-F); group B (*Bacillus cereus* group) was completely excluded (Köberl *et al*., 2011)

[*] According to 16S rRNA gene sequencing.

Genome sequencing of beneficial bacteria

Paenibacillus polymyxa Mc5Re-14, *Streptomyces* sp. Wb2n-11 and *Bacillus amyloliquefaciens* Co1-6 were incubated in flasks for two days (30°C/shaking) in TSB media. 0.5% glycine was added to the media used for the growth of *Streptomyces sp.* Wb2n-11 in order to facilitate the subsequent cell disruption due to minimizing the peptidoglycan crosslinking as mentioned by Nikodinovic *et al*. (2003). Genomic DNA was isolated using the MasterPure™ DNA Purification Kit (Epicentre; Madison, USA) following the manufacturers' protocol. The ONCs were used as starting material and centrifuged at 3000 rpm for 5 min. as the first step. The protocol was expanded through an additional mechanical cell disruption step with glass beads in the ribolyser (FastPrep®-24, MP Biomedicals; Santa Ana, USA) before the addition of Proteinase K. To further increase cell disruption, 80 µl of 1.5% lysozyme (15 mg ml^{-1} lysozyme in 30 mM Tris-HCl and 1 mM EDTA; pH=8) were added and the approaches were subjected to an additional 30 min. incubation step at 37°C while vortexing every 5 min. After applying those additional steps in order to increase the DNA yield, the manufactures' protocol was continued from the incubation step at 65°C for 15 min. After purification, the purity (260/280 and 260/230 ratio) of the DNA samples was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, USA). After 1 µl of TE buffer used as a blank was measured, 1 µl of each sample was analyzed with the spectrophotometer. The concentration of the isolated DNA was determined using the Quant-iT™ PicoGreen® dsDNA quantification kit (Life Technologies; Carlsbad, USA). Therefore, a high range standard curve using the Lambda DNA-standard [100 µg/ml] provided in the kit was created by preparing 400 µl of a 1:50 dilution of the Lambda DNA-standard [100 µg/ml] with 1 x TE buffer. Based on that dilution, further decimal dilutions of the Lambda DNA-standard up to $1*10^{-3}$ were done to reach solutions with Lambda DNA concentrations of 1000 ng/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml. Additionally, a 200-fold dilution of the Quant-iT™ PicoGreen® dsDNA reagent was prepared using 1x TE buffer. The measurement was carried out in black 96 well plates (Greiner Bio-One; Kremsmünster, Austria) in a total reaction volume of 200 µl per well. Standards were measured in two replicates each consisting of 100 µl of one of the Lambda DNA standard dilutions and 100 µl of the diluted Quant-iT™ PicoGreen® dsDNA reagent. For the measurement of the different DNA samples, 1 µl of the isolated DNA was mixed with 99 µl 1 x TE puffer and 100 µl of the diluted Quant-iT™ PicoGreen® dsDNA reagent (Table 7).

Table 7: Measurement conditions to determine the DNA concentrations of isolated antagonistic bacteria with the Quant-iT™ PicoGreen® dsDNA quantification kit. Measurements were conducted in 96 well plates using the microplate reader.

100 µl standard [1000 ng/ml]	100 µl standard [1000 ng/ml]	100 µl standard [1000 ng/ml]	99 µl 1x TE + 1 µl DNA sample1
100 µl standard [100 ng/ml]	100 µl standard [100 ng/ml]	100 µl standard [100 ng/ml]	99 µl 1x TE + 1 µl DNA sample1
100 µl standard [10 ng/ml]	100 µl standard [10 ng/ml]	100 µl standard [10 ng/ml]	99 µl 1x TE + 1 µl DNA sample2
100 µl standard [1 ng/ml]	100 µl standard [1 ng/ml]	100 µl standard [1 ng/ml]	99 µl 1x TE + 1 µl DNA sample2
100 µl 1x TE buffer	100 µl 1x TE buffer	100 µl 1x TE buffer	-

Each DNA sample was measured in duplicates. After adding the Quant-iT™ PicoGreen® dsDNA reagent, the 96 well plate (black-coloured) was covered with a lid to protect it from photo degradation and incubated at room temperature for 5 minutes before performing the measurement with the microplate reader (Infinite® 200 PRO; Tecan; Männedorf, Switzerland). To meet the purity (OD 260/280 ≥ 1.8; OD 260/230 ≥ 1.9) and concentration (200 ng μl^{-1} and min. 10 µg per sample) requirements for sequencing, different isolation approaches were pooled together and obtained DNA was sent for PacBio RS II Sequencing (Pacific Biosciences; Menlo Park, USA) to GATC Biotech (Konstanz, Germany).

Results

Cultivation and characterization of lactobacilli

The first colonies appeared on the R2A agar plates much earlier than colonies on the plates of the other cultivation approaches. The appearance of the colonies was diverse and ranging from small colonies to rather big colonies of different shape and color. Through the Katalase test the formerly 558 isolatates could be reduced to 394 potential lactic acid bacteria (Table 8). The highest number of isolates not belonging to the LAB due to their performance in the Katalase test was obtained from the aerobically cultivated approaches on R2A agar of which the former 168 isolates could be reduced to 60. Finally, 144 isolates were obtained from the three different cabbage samples, 141 isolates belonged to samples derived from *M. chamomilla* L. and 109 attained from *C. officinalis* L. Out of 324 isolates collected in total from the fermentation approaches of the medicinal plants (168 from *M. chamomilla* L. and 156 from *C. officinalis* L.), 250 isolates (141 from *M. chamomilla* L. and 109 from *C. officinalis* L.) were classified as potential LAB (Gram-positive and Katalase-negative) and selected for further characterization based on genomic fingerprinting and 16S rRNA gene sequencing. Moreover, 144 potential LAB isolates were obtained out of 234 *B. oleracea* L. isolates and additionally characterized. Of the potential LAB collection isolated from the medicinal plants, 102 potential LAB were obtained from microaerobic cultivation on MRS agar, 77 and 48 from aerobic and anaerobic incubation of MRS agar, respectively, and 23 isolates from aerobic cultivation on non-selective R2A agar.

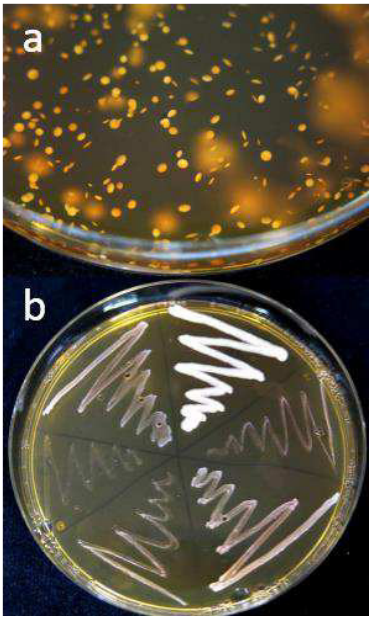

Figure 3: Colonies derived from fermented plant samples performing the poor plating method on MRS agar (a). Dilution streaks of selected colonies derived from the fermented plant samples on MRS agar (b).

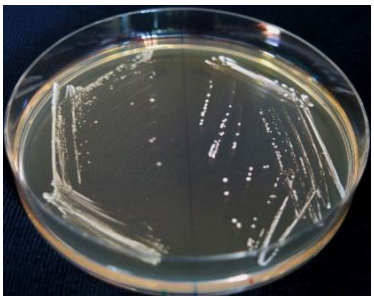

Figure 4: Dilution streaks of potential lactic acid bacteria gained through fermentation.

Figure 5: Negative (left) and positive (right) Katalase test of colonies derived from the fermentation approaches.

The selected cabbage isolates were more equally distributed between cultivation conditions: 44 isolates originated from microaerobic cultivation on MRS agar, 37 from aerobic cultivation on R2A agar, and 37 and 27 isolates were obtained from aerobic and anaerobic cultivation on MRS agar, respectively.

Table 8: Number of colonies derived through different cultivation approaches on R2A and MRS agar transferred to new media for dilution streaking. The last column lists the number of potential LAB after performing the selective Katalase and KOH test.

Cultivation approach	Number of colonies picked for cultivation	Number of potential LAB
KGS R2A aerob	24	0
KKS R2A aerob	24	13
KÖ R2A aerob	24	24
McS R2A aerob	24	0
McF R2A aerob	24	22
CoS R2A aerob	24	0
CoF R2A aerob	24	1
KGS MRS aerob	24	0
KKS MRS aerob	24	24
KÖ MRS aerob	12	12
McS MRS aerob	24	24
McF MRS aerob	24	23
CoS MRS aerob	18	18
CoF MRS aerob	12	12
KGS MRS anerob	12	12
KKS MRS anerob	12	12
KÖ MRS anerob	6	3
McS MRS anerob	12	12
McF MRS anerob	12	12
CoS MRS anerob	12	12
CoF MRS anerob	12	12
KGS MRS pour plating	24	23
KKS MRS pour plating	24	19
KÖ MRS pour plating	24	2
McS MRS pour plating	24	24
McF MRS pour plating	24	24
CoS MRS pour plating	24	24
CoF MRS pour plating	30	30
Total	**558**	**394**

Abbreviations: CoF…*Calendula officinalis* L. Faiyum oasis, CoS…*Calendula officinalis* L. Sekem, McF…*Matricaria chamomilla* L. Faiyum oasis, McS…*Matricaria chamomilla* L. Sekem, KGS…"big" cabbage Sekem, KKS..."small" cabbage Sekem, KÖ…Austrian cabbage.

Comparing the pH values of the different plant samples after three weeks of fermentation (Table 9), the two approaches of *C. officinalis* L. do outstand the others. Their pH with a value of 6.4 (*C. officinalis* L. Sekem) and 6.8 (*C. officinalis* L. Faiyum) was almost neutral, while the values of all other approaches were acidic and in the range of 2.7 (Austrian cabbage) to 3.8 (*M. chamomilla* L. Faiyum).

Table 9: Measured pH values after three weeks of fermentation of different plant samples. Measurements of the fermentation broth were done with a pH Meter.

Sample	Meassured pH
McF	3.8
McS	2.8
CoF	6.8
CoS	6.4
KGS	2.8
KKS	3.0
KÖ	2.7

Abbreviations: CoF…*Calendula officinalis* L. Faiyum oasis, CoS…*Calendula officinalis* L. Sekem, McF…*Matricaria chamomilla* L. Faiyum oasis, McS…*Matricaria chamomilla* L. Sekem, KGS…"big" cabbage Sekem, KKS..."small" cabbage Sekem, KÖ…Austrian cabbage.

Regarding the CFU calculations, most CFUs were counted on the plates cultivated aerobically with R2A as media ($\log_{10}$ CFU ml^{-1} of 7.67) followed by the approach incubated aerobically with MRS agar ($\log_{10}$ CFU ml^{-1} of 7.41). The two approaches incubated anaerobically showed a slightly lower level with $\log_{10}$ CFU ml^{-1} of 7.02 and 7.09 (Table 10).The lowest number of $\log_{10}$ CFU ml^{-1} was examined for the "big cabbage" of Sekem (KGS) cultivated anaerobically with an $\log_{10}$ CFU ml^{-1} of 4.10. Generally, in 3 of all 4 approaches the "big cabbage" of Sekem showed the lowest $\log_{10}$ CFU ml^{-1}, with an average $\log_{10}$ CFU ml^{-1} of 5.71. Interestingly, the highest $\log_{10}$ CFU ml^{-1} was observed for the fermented plant samples of *M. chamomilla* L. with $\log_{10}$ CFU ml^{-1} of 8.38 for the plant samples derived from Faiyum oasis and $\log_{10}$ CFU ml^{-1} of 8.17 for all the four approaches harvested from Sekem. The $\log_{10}$ CFU of the Austrian cabbage ($\log_{10}$ CFU ml^{-1} of 7.27) remained within the values analyzed for the approaches of *C. officina*lis L. with $\log_{10}$ CFU ml^{-1} of 7.25 for the samples derived from Faiyum oasis and of 7.59 for the approaches sampled at Sekem. A graphical comparison of the different CFU ml^{-1} can be gathered from Figure 6, where the calculated CFU ml^{-1} of the different plant samples after fermentation and subsequent cultivation under varying cultivation conditions are evident.

Table 10: Colony forming unit per ml of different fermentation approaches.

Log_{10} CFU ml^{-1}	CoF	CoS	McF	McS	KGS	KKS	KÖ	Average
R2A aerob	8.26	8.25	7.96	8.54	6.83	6.45	7.38	**7.67**
MRS aerob	7.05	7.46	8.55	8.09	6.46	6.93	7.34	**7.41**
MRS anaerob	6.92	7.31	8.62	8.17	4.10	6.71	7.32	**7.09**
MRS pour plating	6.77	7.32	8.41	7.87	5.44	6.75	7.05	**7.09**
Average	**7.25**	**7.59**	**8.38**	**8.17**	**5.71**	**6.71**	**7.27**	

Abbreviations: CoF…*Calendula officinalis* L. Faiyum oasis, CoS…*Calendula officinalis* L. Sekem, McF…*Matricaria chamomilla* L. Faiyum oasis, McS…*Matricaria chamomilla* L. Sekem, KGS…"big" cabbage Sekem, KKS..."small" cabbage Sekem, KÖ…Austrian cabbage.

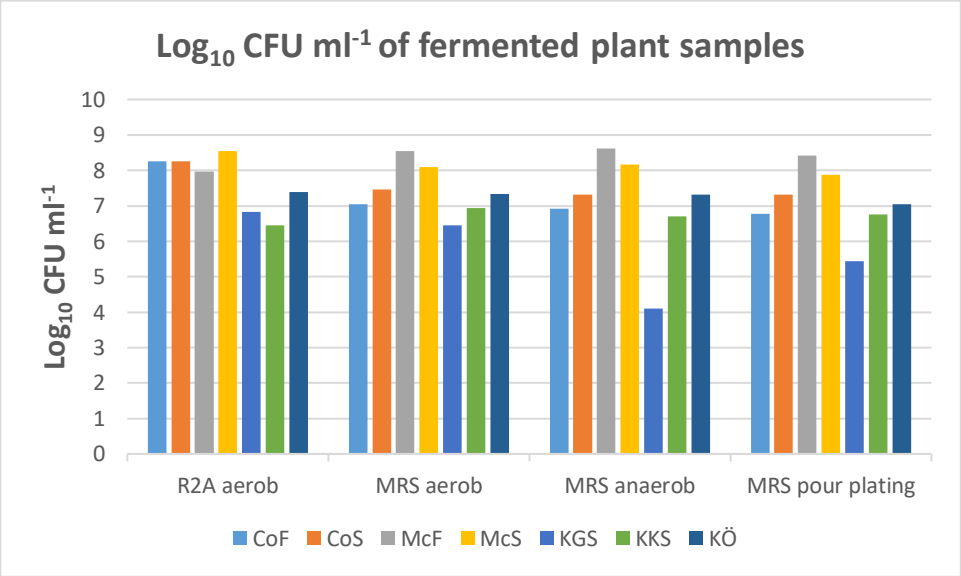

Figure 6: Calculated log_{10} CFU ml-1 of fermented plant samples under different culture conditions.
Abbreviations: CoF…*Calendula officinalis* L. Faiyum oasis, CoS…*Calendula officinalis* L. Sekem, McF…*Matricaria chamomilla* L. Faiyum oasis, McS…*Matricaria chamomilla* L. Sekem, KGS…"big" cabbage Sekem, KKS..."small" cabbage Sekem, KÖ…Austrian cabbage.

In vitro antagonistic potential of isolated lactic acid bacteria towards plant and human pathogens

Regarding the antagonistic potential of the isolated lactic acid bacteria, the dual culture assay achieved no antagonistic potential of all tested isolates, an inhibition zone was detected only around the positive controls. Figure 7 illustrates the results of lactic acid bacteria (Figure 7-a and Figure 7-c) in comparison to the positive controls (Figure 7-b and Figure 7-d) in the dual culture assay against selected soil-borne pathogens. Figure 7-a and Figure 7-b display the results of the potential lactic acid bacteria and the positive controls against *Verticillium dahliae.*

While in Figure 7-a, the fungus was spread over the whole plate, only sparing the spot where the lactic acid bacteria were plotted, the fungus was strongly repressed in growth in Figure 7-b. Only the space between the plotted isolates was colonized sparely by the fungus. The test against *Fusarium culmorum* revealed a similar picture. Against the test strains derived through fermentation the fungus was growing unhampered while showing almost inhibited growth on the control plates.

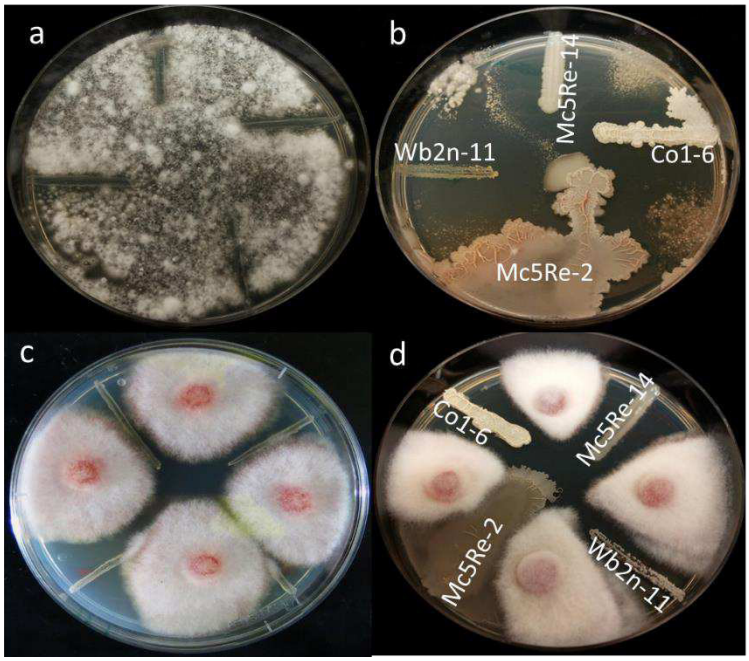

Figure 7: Dual culture assay on Waksman agar to test the antagonistic potential of potentially lactic acid bacteria. Isolates tested against *Verticillium dahliae* V25 (a). Positive controls tested against *Verticillium dahliae* V25 (b). Antagonistic test of isolates against *Fusarium culmorum* E1 (c). Positive controls tested against *Fusarium culmorum* E1 (d).

Only the isolate Mc5Re-2 of the positive control was not able to limit the growth of the fungus as can be seen in Figure 7-d, where the mycelium of *Fusarium culmorum* extended as far as the bacterial spreading reached. Against the three tested potential human pathogens (*Staphyloccocus aureus*, *Escherichia coli* and *Salmonella typhimurium*), neither the test strains nor one of the potential LAB showed growth inhibition.

Fingerprint analyses of isolated lactic acid bacteria from various plant sources using ARDRA and rep-PCR

Based on the restriction digest pattern generated with the enzyme *Hha*I (one of the gels can be seen in Figure 8), the isolates of the fermentation could be divided into five distinct ARDRA groups (A-E). The two groups B and D containing the major number of isolates with 196 and 173 could therefore be termed as main groups. The distribution of the number of isolates based on their ARDRA digestion pattern and the plants from which the isolates were obtained can be gathered from Table 12. The restriction digestion patterns are comparable with the results derived through sequencing and the comparison of the virtual electrophoresis (Figure 9**,** Table 11) patterns created with the program pDRAW32 (AcaClone software) of the closest database matches of the sequenced samples. In the case of the isolate 280 with its closest

database matches *Lactobacillus casei* (NR_075032.1) and *Lactobacillus paracasei* subsp. *tolerans* (accession number: NR_041054.1) the enzyme *Hha*I cuts two times thereby creating fragments with the size of 52, 299 and 537.

For the isolate 287, where *Lactobacillus nenjiangensis* was found as closest database match (accession number: NR_125563.1), *Hha*I had three points of attac and generated four fragements. ARDRA group A only contained one isolate obtained from fermentated cabbage and had a band pattern different from the other samples in the virtual electrophoresis. Sequencing analysis classificated the strain as *Lactobacillus fabifermentans* (identity: 99%, accession number: NR_113339.1). Samples from the ARDRA groups D and E were derived from the two medicinal plants except for one sample of group D coming from cabbage. The small groups A and C in contrast are composed only of samples derived from fermented cabbage. From the biggest ARDRA group B, 128 isolates out of 196 were obtained from cabbage plant material, 64 from *M. chamomilla* L. and only 4 isolates from *C. officinalis* L. (Figure 10).

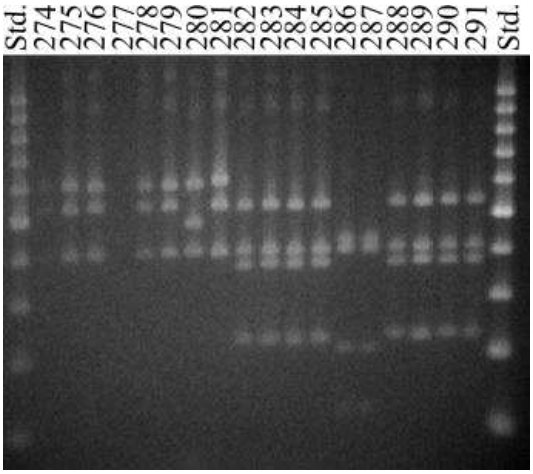

Figure 8: Different band patterns of isolates derived through restriction with the enzyme *Hha*I performing ARDRA.

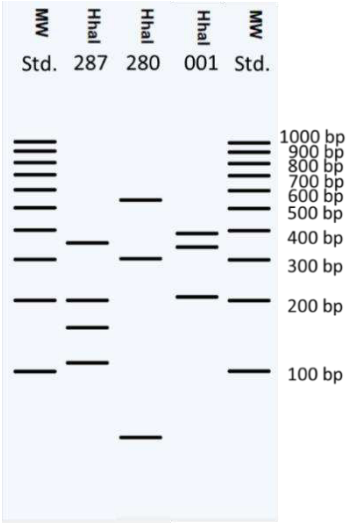

Figure 9: Virtual electrophoresis performance of different DNA sequences gathered from sequencing of isolates of potential LAB and cut with the enzyme *Hha*I.

Table 11: Restriction analysis of sequenced fermentation samples after virtual *Hha*I digestion with the program pDRAW32.

Sample	Total length	Number of cuts	Cut position	Fragment sizes
1: KKS1-1	938	2	207 547	207 340 391
212: McS3-12	887	1	537	350 537
280: KÖ4-12	888	2	537 589	52 299 537

154: CoS2-11	876	2	157 530	157 346 373
287: McS4-7	818	3	354 555 664	109 154 201 354
316: McS4-7	889	1	536	353 536
233: CoF3-9	958	2	163 536	163 373 422
218: CoS3-6	897	1	545	352 545
353: CoF4-1	952	2	157 530	157 373 422
183: KGS3-10	887	1	535	535 352

Abbreviations: CoF…*Calendula officinalis* L. Faiyum oasis, CoS…*Calendula officinalis* L. Sekem, McF…*Matricaria chamomilla* L. Faiyum oasis, McS…*Matricaria chamomilla* L. Sekem, KGS…"big" cabbage Sekem, KKS…"small" cabbage Sekem, KÖ…Austrian cabbage.

Table 12: Number of isolates based on the different band patterns derived through ARDRA using the restriction enzyme *Hha*I.

ARDRA Group	Number of isolates
A	1
B	196
C	5
D	173
E	8
unclassified*	11

***no band patterns visible on the gels**

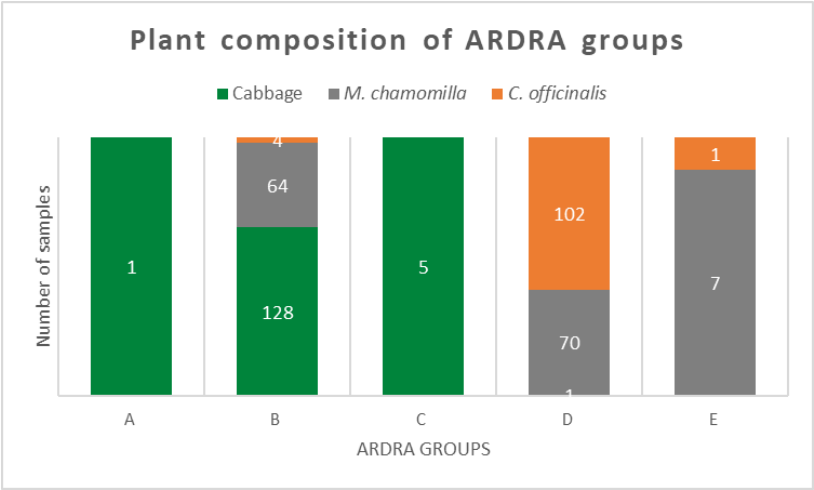

Figure 10: Distribution of plant sources of ARDRA groups based on their performance in electrophoresis gels.

Through alignment of the sequenced 16S rRNA gene sequences with the BLAST algorithm against the reference RNA sequence database, all ten sequenced isolates could be classified as lactic acid bacteria (Table 13). The ten sequences shared all a similarity of 99% with their closest database match of the reference sequence RNA (refseq_RNA) database. All three samples sequenced from the ARDRA group D belonged to the genus *Enterococcus*, a member of the order of *Lactobacillales*, the other seven isolates were of the genus *Lactobacillus*.

Table 13: Results of the sequencing of selected isolates belonging to different ARDRA groups aligned to the reference RNA sequence database using the BLAST algorithm.

ARDRA group	Sample	Strain	Identity	Accession of closest match
A	1	*Lactobacillus fabifermentans*	99%	NR_113339.1
B	183	*Lactobacillus pentosus* *Lactobacillus plantarum*	99% 99%	NR_029133.1 NR_075041.1
B	212	*Lactobacillus pentosus* *Lactobacillus plantarum*	99% 99%	NR_029133.1 NR_075041.1
B	218	*Lactobacillus coryniformis* subsp. *torquens*	99%	NR_029018.1
B	316	*Lactobacillus brevis*	99%	NR_075024.1
C	280	*Lactobacillus casei* *Lactobacillus paracasei* subsp. *tolerans*	99% 99%	NR_075032.1 NR_041054.1
D	154	*Enterococcus casseliflavus*	99%	NR_102793.1
D	233	*Enterococcus casseliflavus*	99%	NR_102793.1
D	353	*Enterococcus casseliflavus*	99%	NR_104560.1
E	287	*Lactobacillus nenjiangensis*	99%	NR_125563.1

The fingerprint pattern of the individual samples derived through rep-PCR with the BOX_A1 primer looked almost all the same, not allowing a distinction and classification of the different isolates (Figure 11). There where around six bands visible for each amplified isolate.

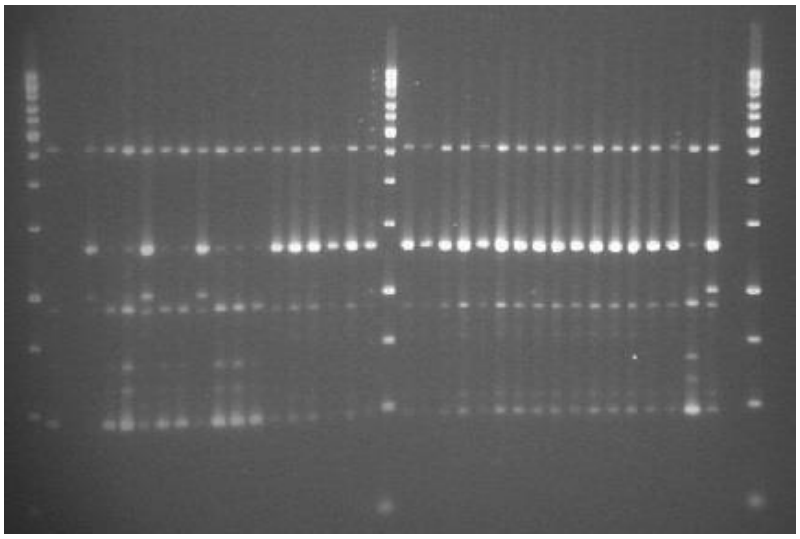

Figure 11: BOX-gel of fermentation isolates from different plant samples amplified with the BOX_A1 primer and visualized under the UV transilluminator. As a standard the GeneRuler™ 1 kb DNA Ladder Ready-to-Use was used.

Cultivation-independent characterization: bacterial colonization of native and fermented plant material

To have a detailed view of the starting material, the phyllosphere microbiomes of *M. chamomilla* L. and *C. officinalis* L. leaves were studied for the ectosphere (outer surfaces) and endosphere (inner tissues) using 16S rRNA gene amplicon sequencing. Overall, after excluding sequences assigned to chloroplasts, mitochondria and archaeal 16S rRNA, and after removing single- and doubletons, 1399 bacterial operational taxonomic units (OTUs, 16S

rRNA genes at 97% similarity cut-off) were identified, with up to 241 OTUs per ectosphere and 89 OTUs per endosphere sample. Alpha diversity indicated by Shannon index showed that for both plants the bacterial diversity in the leaf endosphere was significantly lower than in the ectosphere (Figure 12-a). In neither of the two investigated leaf communities, statistically significant differences occurred in the diversity between *M. chamomilla* L. and *C. officinalis* L. A principal coordinate analysis (PCoA) based on the distance matrix calculated using the Bray- Curtis metric showed a clear sample separation of ectosphere and endosphere samples ($p = 0.001$) into three groups (Figure 12-b). The endosphere samples of both plants were highly similar and clustered closely together, while the ectosphere samples were just slightly overlapping, showing that although the bacterial diversity in the ectosphere of the two plants was similar, they had a differing bacterial composition (82 significantly different $OTUs_{0.97}$). The composition of the bacterial communities at phylum level was dominated by *Proteobacteria*, *Firmicutes* and *Actinobacteria*, comprising together more than 90% of relative abundance in the ectosphere of both medicinal plants. Additionally, *Chloroflexi* and *Bacteroides* were found in both plant ectospheres in a relative abundance over 2%. At class level, *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Bacilli* and *Actinobacteria* were the major representative taxa in the ectosphere communities (Figure 13). The leaf endosphere of both plants was highly dominated by *Proteobacteria* (~95% in both endosphere communities). *Firmicutes* and *Actinobacteria* were also found in both plant endospheres (>1%), however in much lower abundance. The *Proteobacteria* inhabiting the inner plant tissue could be divided into *Alpha-*, *Beta-* and *Gammaproteobacteria*, whereby *Gammaproteobacteria* were highly predominant (~97% of classified *Proteobacteria* and ~91% of the total endophytic microbiome). *Bacilli* were detected in both plant endospheres in a relative abundance >1%. *Bacilli* assigned to *Lactobacillales* were detected in the leaf ecto- as well as endosphere of both medicinal plants in relative abundances between 0.7% and 0.3%, whereby no clear habitat preference was discernible.

Bacterial diversity in the fermentation approaches measured by the Shannon diversity index was significantly lower than in the original leaf ectospheres ($p = 0.003$) and similar to the diversity of the leaf endospheres ($p = 0.915$). Concerning the bacterial community composition in *M. chamomilla* L. and *C. officinalis* L. during six weeks of fermentation, the predominant phyla in both approaches were *Proteobacteria* and *Firmicutes*. Members of *Actinobacteria* and *Bacteroidetes* were more prevalent in *M. chamomilla* L. than in *C. officinalis* L., with a maximum of 3.5% and 5.6% of the total community in the chamomile

fermentation and 1.4% and 0.6% in the *Calendula* fermentation, respectively. The phyla *Acidobacteria*, *Verrucomicrobia*, *Chloroflexi*, *Nitrospirae*, *Tenericutes*, *Planctomycetes*, *Gemmatimonadetes*, *AD3* and *Thermi* were further found in both fermentation approaches, however in very low abundances (<0.1%). *TM6* were only detected in the earlier stage (week 1 to 3) of the *Calendula* fermentation. At order level, the most dominant taxa were *Pseudomonadales*, *Burkholderiales* and *Enterobacteriales*, followed by *Clostridiales*, *Bacillales* and *Lactobacillales* (Figure 14). As a reflection of the microbial community dynamics, the relative abundances of bacterial OTUs varied between plant species and over the fermentation period. All taxa which were detected in the fermentation approaches were also found in the freshly collected plant samples, but sometimes in quite subordinate presence, like for example the lactic acid bacteria (<0.8% in the phyllosphere microbiomes). *Alphaproteobacteria* which were identified as relatively dominant members of the ectospheric leaf communities with 25.6% and 26.1% of relative abundance were hardly detectable during fermentation (<0.7%). The relative abundance of *Firmicutes* increased in both approaches over time, whereby *Lactobacillales* did not significantly change after the initial increase within the first week of fermentation. *Actinomycetales* and *Sphingobacteriales* were more prevalent in the fermentation of *M. chamomilla* L., with an increase in relative abundance over time.

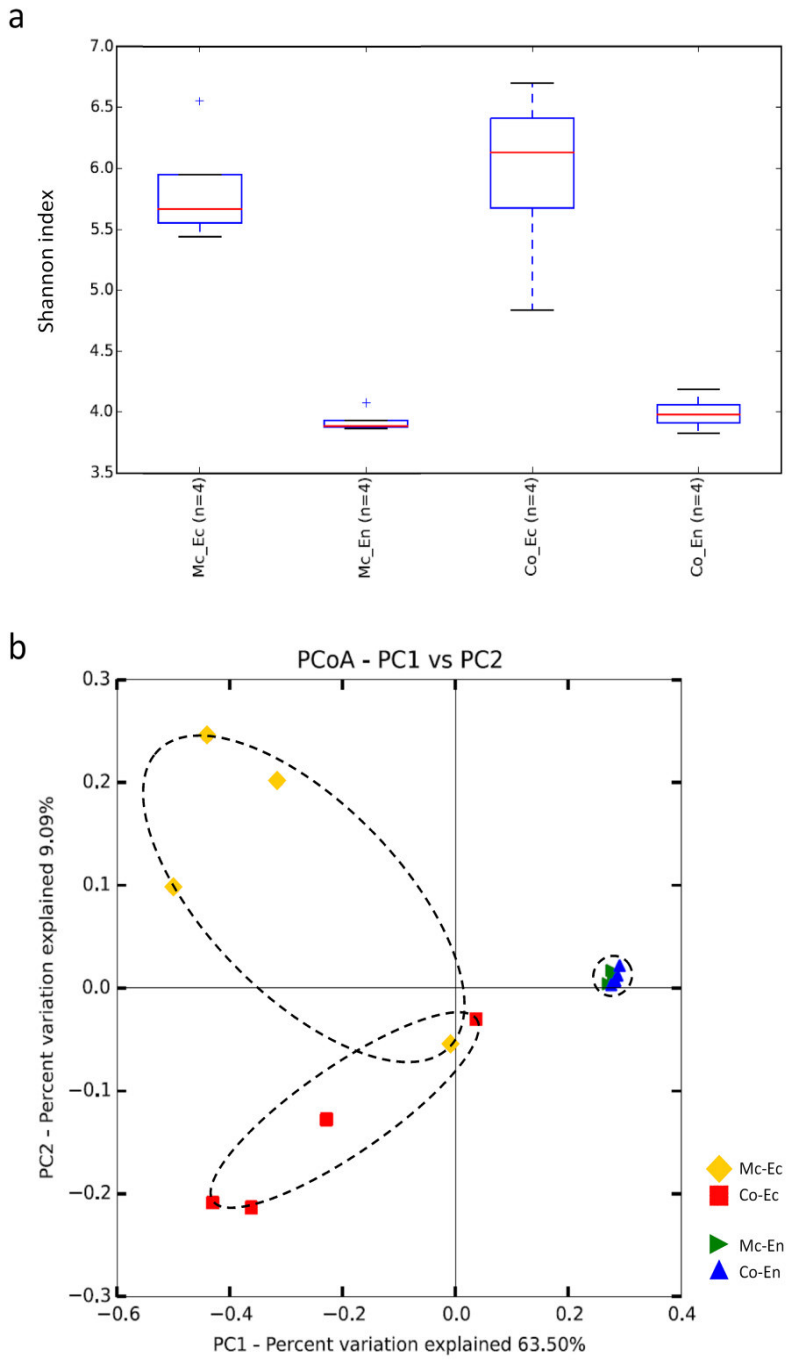

Figure 12: Shannon diversity at a genetic distance of 3% (a) and principal coordinate analysis (PCoA) biplot based on Bray-Curtis dissimilarity (b) of the bacterial communities colonizing the leaf ecto- (Ec) and endosphere (En) of *Matricaria chamomilla* L. (Mc) and *Calendula officinalis* L. (Co) grown under desert-farming conditions in Egypt. Data were ascertained by 16S rRNA gene profiling in four independent replicate samples per plant species.

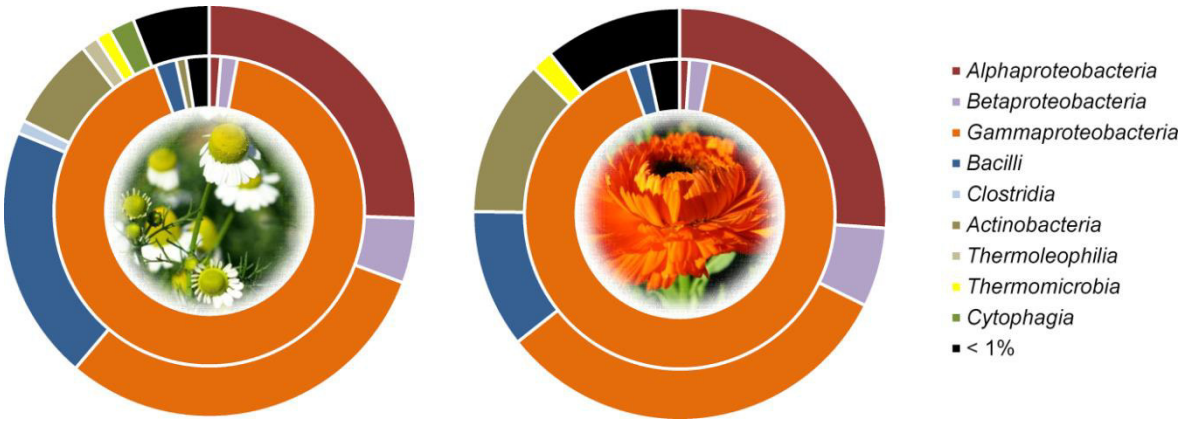

Figure 13: Taxonomic composition of the bacterial phyllosphere microbiome of *Matricaria chamomilla* L. and *Calendula officinalis* L. grown under desert-farming conditions in Egypt. The outer circles represent the ectosphere colonization, while the inner circles show the class distribution within the leaf endophytes. Mean values of four independent replicate samples subjected to 16S rRNA gene profiling are depicted for each plant species.

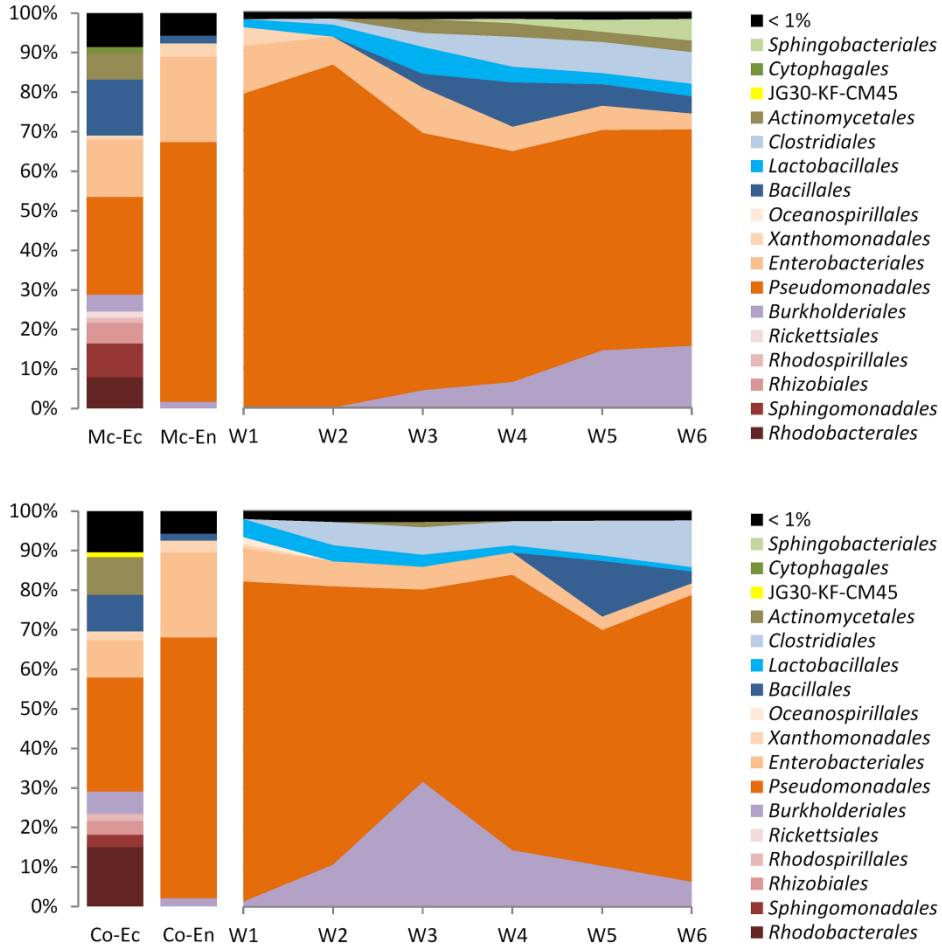

Figure 14: Order composition of the bacterial microbiome in fresh leaves of *Matricaria chamomilla* L. (Mc) and *Calendula officinalis* L. (Co) and dynamics over a six weeks fermentation period (W1-W6). First two columns represent the ectospheric (Ec) and endospheric (En) leaf colonization. Mean values of four independent replicate samples subjected to 16S rRNA gene profiling are depicted for each plant species.

Regarding the dynamic of the LAB, *Lactobacillales* in the fermentation approach of *M. chamomilla* L. reached their highest relative abundance with 6.8% after three weeks of fermentation. In the approach with *C. officinalis* L., they reached their maximum abundance with 4.6% already after one week and gradually decreased in the course of fermentation (Figure 14). Looking at the taxonomic distribution within the LAB, members could be divided into six families: *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Streptococcaceae*, *Carnobacteriaceae* and *Aerococcaceae*. All of them were found in the original plant samples and during fermentation. *Enterococcus* belonging to the *Enterococcaceae* was the most dominant genus during both medicinal plant fermentations, with a maximum relative abundance of 67.9% after four weeks in the chamomile fermentation and 59.5% after five weeks in the *Calendula* fermentation (Figure 15). In the fresh leaves, *Enterococcus* accounted for 26.5% and 18.8% of relative abundance in the ectospheric LAB communities and nearly half (48.8% and 51.5%) of the endophytic LAB. However, one needs to consider that the presence of *Lactobacillales* within the total bacterial microbiome was much lower in the unfermented samples. Other members of *Enterococcaceae* (genera *Tetragenococcus* and *Vagococcus*) were exclusively detected in the ectosphere of the chamomile, but disappeared during its fermentation. Besides *Enterococcus*, *Pediococcus* belonging to the *Lactobacillaceae* was identified as dominant genus throughout the chamomile fermentation (max. 32.2% after three weeks), while it played a minor role in the marigold fermentation (max. 1.0% after three weeks). *Pediococcus* (*Lactobacillaceae*) was detected as ecto- and endophyte in both medicinal plants. *Lactobacillus* (*Lactobacillaceae*) was present in the leaf ectospheres and in the fermentation approaches, reaching its highest relative abundance with 4.2% after six weeks in the chamomile approach and with 3.0% after three weeks in the marigold approach. While *Lactobacillaceae* were a dominant group in the chamomile fermentation, *Leuconostocaceae* (genera *Leuconostoc* and *Weissella*) and *Streptococcaceae* (genus *Lactococcus*) were identified as dominant LAB in the fermentation of *Calendula*. The genus *Streptococcus* (*Streptococcaceae*) was detected in both ectosphere communities, but was not present in the endospheres and during fermentation. *Desemzia* belonging to the *Carnobacteriaceae* was the third dominant genus in the early fermentation of *M. chamomilla* L. diminishing over time, while it occurred in general lower relative abundance but stable throughout the marigold fermentation. *Trichococcus* (*Carnobacteriaceae*) was detected in the leaf ectosphere of both plants, but not in the inner tissue and barely in the fermentation approaches. *Aerococcaceae* (genera *Marinilactibacillus*, *Aerococcus* and *Facklamia*) were quite present in the original leaf samples (30.3% in the *M.*

chamomilla L. ectosphere and 9.1% in the *C. officinalis* L. ectosphere), but revealed a subordinate presence within the course of fermentation.

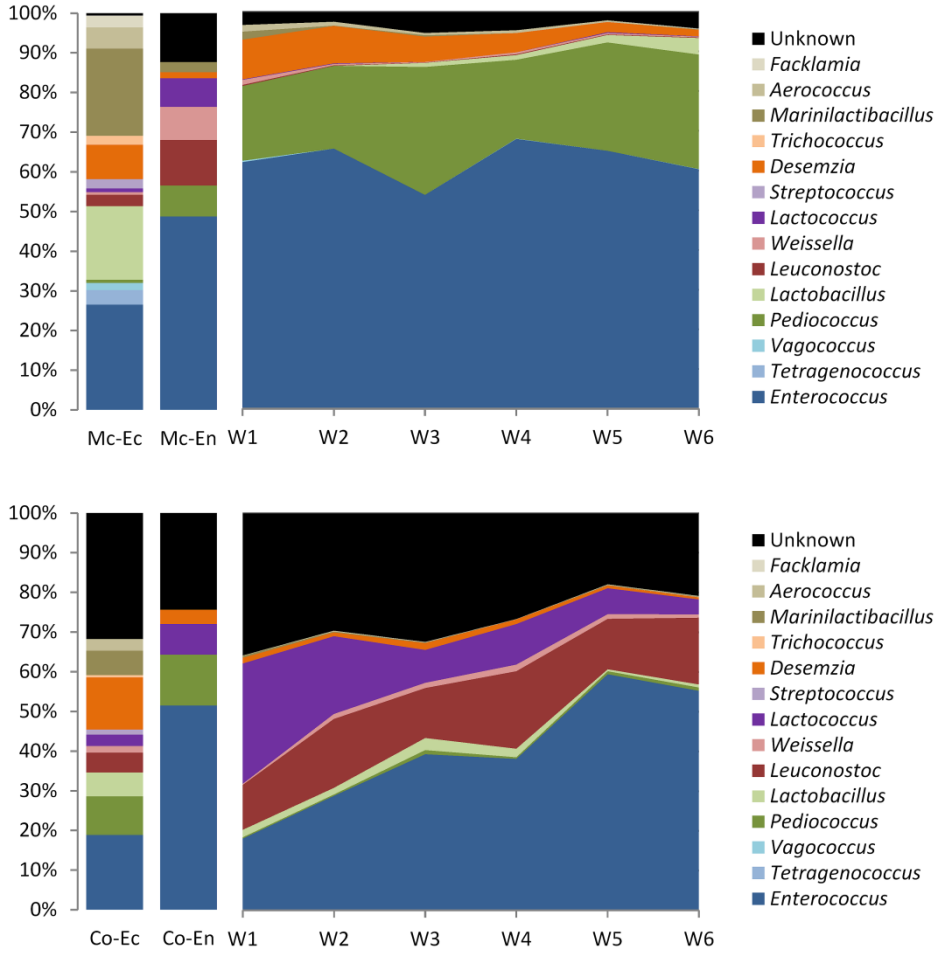

Figure 15: Genus structure of the *Lactobacillales* communities in fresh leaves of *Matricaria chamomilla* L. (Mc) and *Calendula officinalis* L. (Co) and dynamics over a six weeks fermentation period (W1-W6). First two columns represent the ectospheric (Ec) and endospheric (En) leaf colonization. Mean values of four independent replicate samples subjected to 16S rRNA gene profiling are depicted for each plant species.

After amplification with specific primers for *Gammaproteobacteria* and pseudomonads (Figure 16) and single strand conformational polymorphism (SSCP) analysis of different cabbage samples (small, big and Austrian), band patterns of the four replicates of each cabbage showed rather similar patterns, while the band patterns of the various cabbage samples looked different. The two pseudomonads samples of the small and big cabbage were an exception from this as they both looked quite similar. The big cabbage although had some additional bands in the upper part of the gel which can be seen only slightly in the four replicates of the small cabbage. Furthermore, some bands which occurred in all four replicates of one cultivar did not show up in the other two cabbage variants. There were also bands which were unique in all the samples tested and occurred in just one sample of all approaches.

This was for example true for the relatively thick band in the upper part of the gel of the last replicate of the big cabbage from the *Gammaproteobacteria* community. In the Austrian cabbage, two samples out of the four individual sample approaches from the pseudomonads community seemed to contain similar genera varying from the other two samples. Looking at the intensity of the bands, some of them appeared as rather thick bands compared to other bands which are only slightly visible. Especially some bands of the *Gammaproteobacteria* in the Austrian cabbage appeared as the thickest bands of the whole gel.

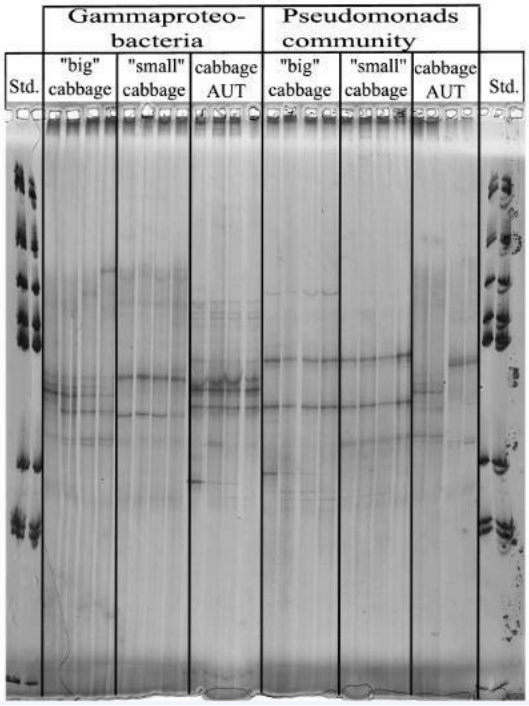

Figure 16: 16S rRNA PCR-SSCP patterns of fresh cabbage from different cabbage heads. Two of them ("small" cabbage and "big" cabbage) from Sekem farms and one cabbage head from Austria (cabbage AUT). PCR amplification was done with primers specific for *Gammaproteobacteria* and pseudomonads.

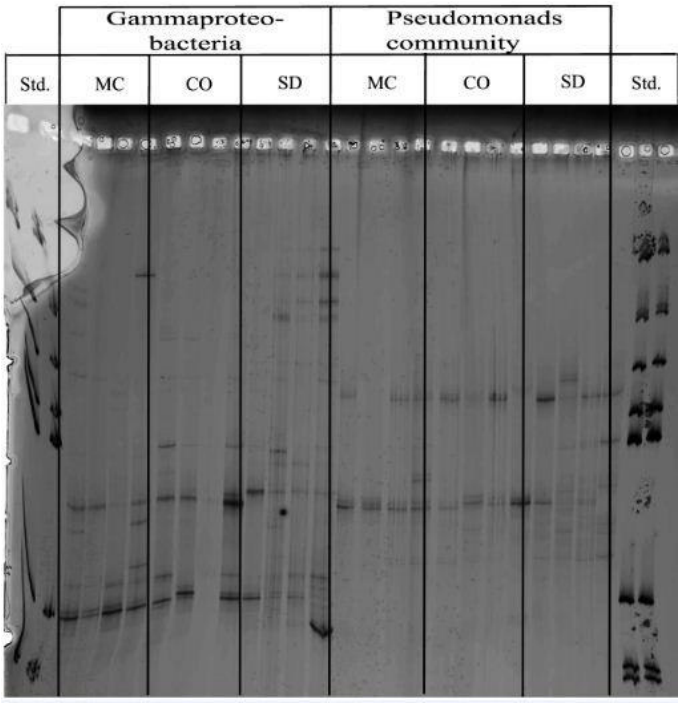

Figure 17: 16S rRNA PCR-SSCP patterns of conserved phyllosphere-samples of medicinal plants collected from Sekem farms. PCR amplification was done with primers specific for *Gammaproteobacteria* and pseudomonads. MC…*M. chamomilla* L.; CO…*C. officinalis* L., SD…*S. distichum* Schumach. and Thonn.

All four individual samples regarding the *Gammaproteobacteria* and the pseudomonads of the medicinal plants (Figure 17) looked again rather similar. In contrast to the cabbage samples, the band patterns among the different plants varied less, making it look that in every plant the same bacteria were more abundant. The gel of the medicinal plants regarding the pseudomonads showed two main intense band lines emerging in almost all of the three medicinal plant samples. Very eye-catching was the individual thick band in the last sample of the *S. distichum* Schumach. and Thonn. in the gel displaying the *Gammaproteobacteria* community. Unfortunately, although attempts were undertaken in reamplifying and sequencing, it was not possible to explore which genus/species was behind that band.

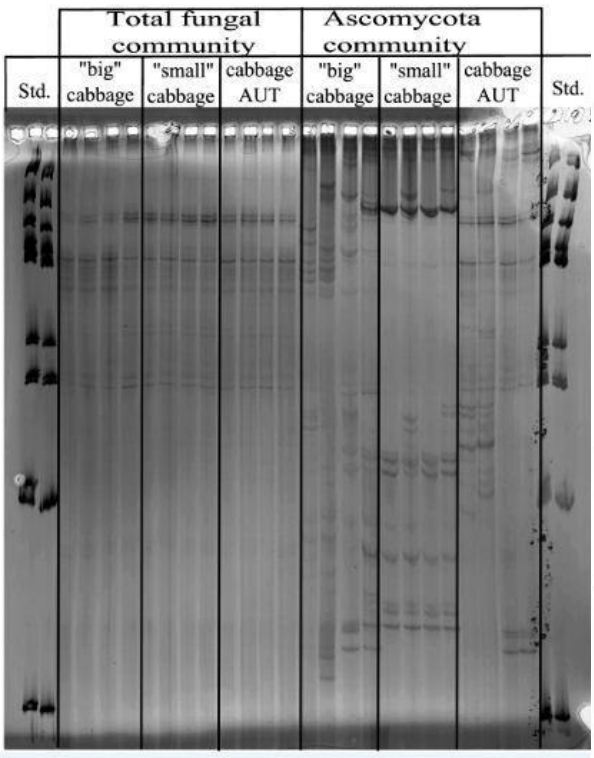

Figure 18: ITS PCR-SSCP patterns of fresh cabbage from different cabbage heads. Two of them ("small" cabbage and "big" cabbage) from Sekem farms and one cabbage head from Austria (cabbage AUT). PCR amplification was done with primers specific for the *Ascomycetes* and fungal community.

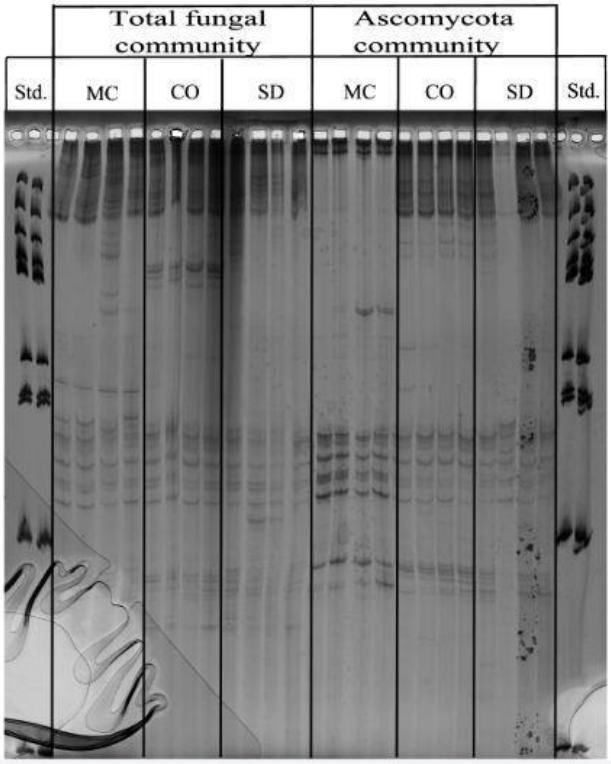

Figure 19: ITS PCR-SSCP patterns of conserved phyllosphere-samples of medicinal plants collected from Sekem farms. PCR amplification was done with primers specific for the *Ascomycetes* and fungal community. MC…*Matricaria chamomilla* L.; CO…*Calendula officinalis* L., SD…*Solanum distichum* Schumach. and Thonn.

Looking at the band patterns of the total fungal communities of the cabbage (Figure 18), the two Egyptian and the Austrian cabbage samples looked rather similar, even more, in all three different cabbage samples no single band varied from the other bands. Only in the intensity of the different bands a difference could be seen. Strikingly, all the bands of the total fungal community were located in the upper part of the gel. Looking at the *Ascomycota* community of cabbage, the gel performance of the Egyptian "big" cabbage differed strongly from the patterns of the other two plant samples and also each of the four individual replicates of the "big" cabbage looked rather different from each other (Figure 18). The lowest bands of the third and fourth individual samples of the big and the Austrian cabbage looked quite similar, probably representing the same genera/species. The four replicates of the *Ascomycota* of the small cabbage all have the same bands in common, the second and fourth sample of the four replicates display some additional bands beside the ones occurring in all samples. In the Austrian cabbage, the upper bands of the *Ascomycota* community reveal a similar band pattern while in the lower part of the gel the first two replica samples differ from the other two (third and fourth) samples. Regarding the total fungal communities of the medicinal plants (Figure 19), the lower part of the gel appeared quite similar in all three medicinal

plants. Furthermore, some bands occurred in all four replicates of one plant, but were not visible in the other two. An example thereof can be seen in the four *M. chamomilla* L. replicates, showing all a unique band just above the third band pattern of the standard. Some bands were also different from the individual samples of the same medicinal plant, this was true for the third sample of *M. chamomilla* L. where five additional bands in the upper part could be identified. In general, a greater number of bands were visible in *M. chamoilla* and *C. officinalis* L. compared to *S. distichum* Schumach. and Thonn. For the *Ascomycota* community of the medicinal plants (Figure 19) the band patterns of all three plants looked again quite similar. An exception were the two rather intense bands of the third and fourth replicates from the medicinal plant *M. chamomilla* L. in the upper part of the gel. An additional band could also be seen in the second replica of *S. distichum* Schumach. and Thonn., accompanying with the missing bands in the lower part of the gel for this replicate. SSCP-PCRs did not work out for the phyllospheric ecto/endosphere samples as well as for the fermentation approaches, therefore no further data of the colonization of the different plants regarding SSCP-gel analysis could be investigated.

Reamplification of the thick bands cut out from the SSCP-gels gave only moderate success, as only a few of them showed bands on the control gel (Figure 20), while most of them were not reamplified. For the bands where the sequencing succeeded, in turn, no significant similarity was found when running the NCBI BLAST algorithm. All matches referred only to predicted hypothetical protein sequences. Four bands cut out of the gel of the total fungal community from the fresh cabbage samples delivered the hypothetical protein EXF-994 of *Wallemia ichthyophaga* (accession number XM_009272224.1) as the closest database match with a similarity of 96%. Another sample from the fresh cabbage gathered from the *Ascomycota* community SSCP gel resulted a hypothetical protein of *Fomitiporia mediterranea* as closest database match. With an identity of 100% but a small query cover of only 35% and 41%, a hypothetical protein of *Bipolaris sorokiniana* was found as database match of the fungal community SSCP gel of two conserved phyllosphere samples of *M. chamomilla* L. Another match with a higher query cover of 96% from a phyllosphere sample of *M. chamomilla* L. again derived a hypothetical protein of *Bipolaris sorokiniana* as closest database match. Two

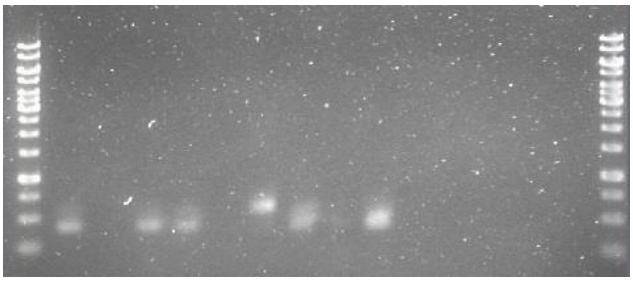

Figure 20: Control gel of cut out and reamplified bands from the SSCP gel showing attempts of reamplifying samples of the pseudomonads from the medicinal plants.

samples of *M. chamomilla* L. one from the total fungal community SSCP gel and the other from the *Ascomycota* SSCP gel revealed *Setosphaeria turcica* as closest database match.

In situ visualisation of bacterial inhabitants on native and fermented plant samples

Phyllosperic plant material as well as the rhizosphere of native and fermented plant material was analyzed for their colonization with different bacterial communities using fluorescence in situ hybridization and confocal laser scanning microscopy. On the conserved roots of *M. chamomilla* L. only a low abundance of *Rhizobiales* was detected (Figure 21). Thereby, *Rhizobiales* (stained yellow) were colonizing a substrate in the root system of *M. chamomilla* L. The bacteria were arranged equally distributed on the substrate but not forming microcolonies. Other not classified bacteria were detected rather vague in red. When looking at other parts of the root system, a high colonization with other *Eubacteria* (red) was observed, forming a dense braid traversing parts of the root (Figure 22).

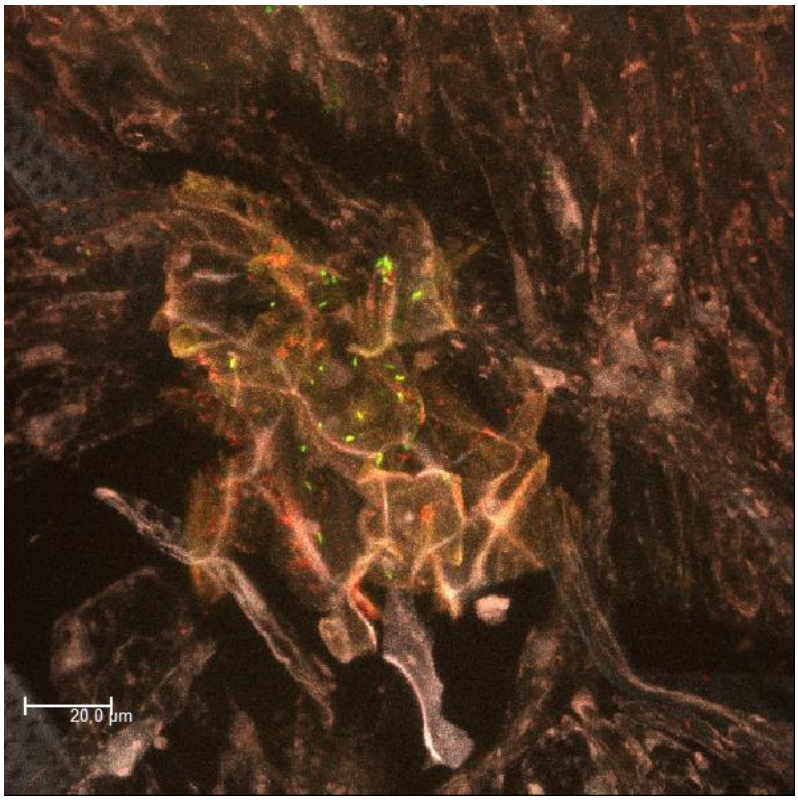

Figure 21: Visualization of FISH-labeled *Rhizobiales* (yellow) colonizing a substrate on the root area of *Matricaria chamomilla* L. Taxonomically not classified bacteria are shown in red.

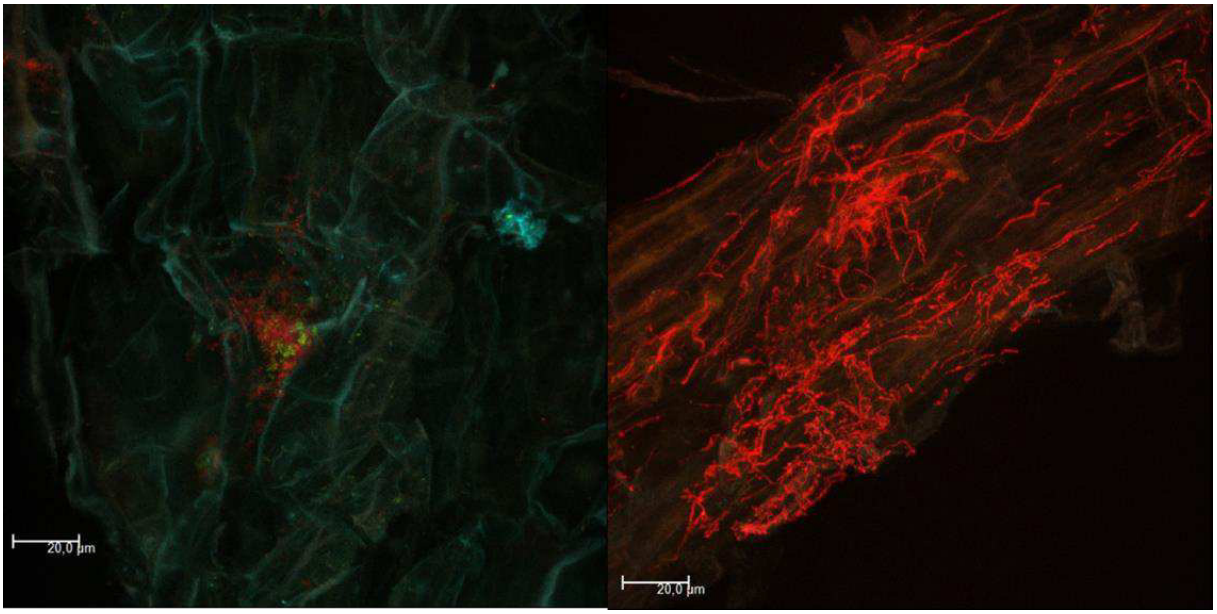

Figure 22: CLSM image of taxonomically not further classified *Eubacteria* (red) traversing root parts of the medicinal plant *Matricaria chamomilla* L.

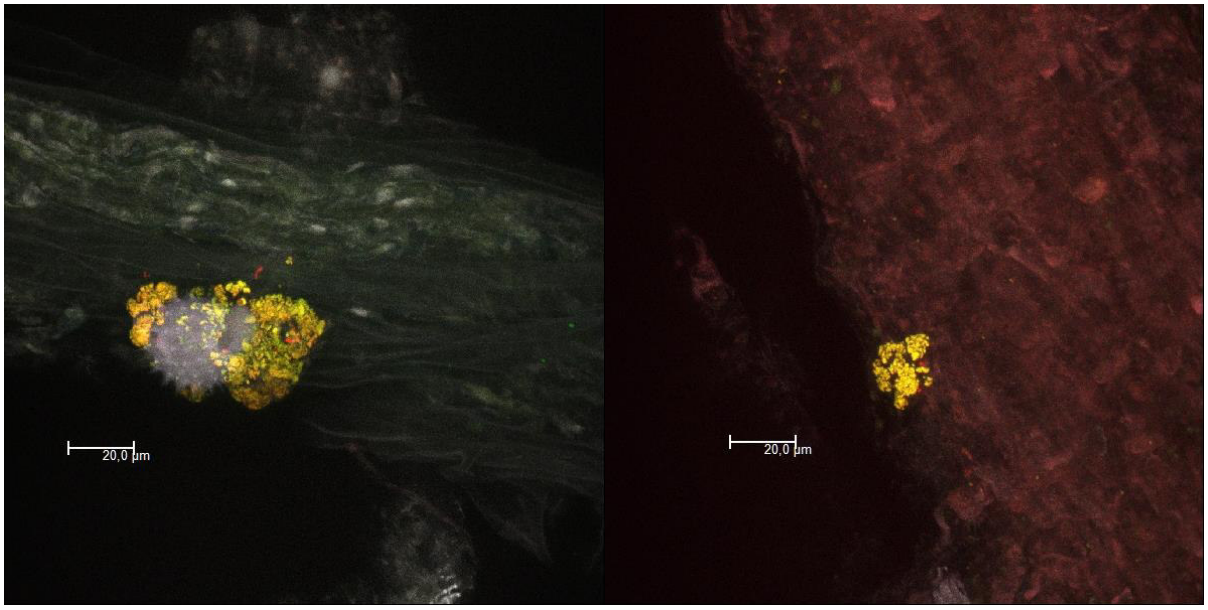

Figure 23: *Gammaproteobacteria* (yellow) on unfermented blossoms of the medicinal plant *Calendula officinalis* L. coloured with fluorescence probes and visualized under the confocal laser scanning microscope. *Gammaproteobacteria* surrounding a pollen grain in dense clouds (left). *Gammaproteobacteria* on not further characterized parts of the blossom (right).

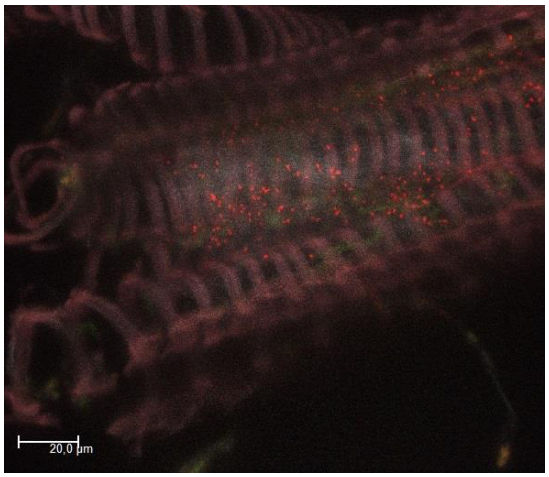

Figure 24: Taxonomically not further characterized *Eubacteria* (red) on fermented leaves samples of *Calendula officinalis* L. visualized with the confocal laser scanning microscope.

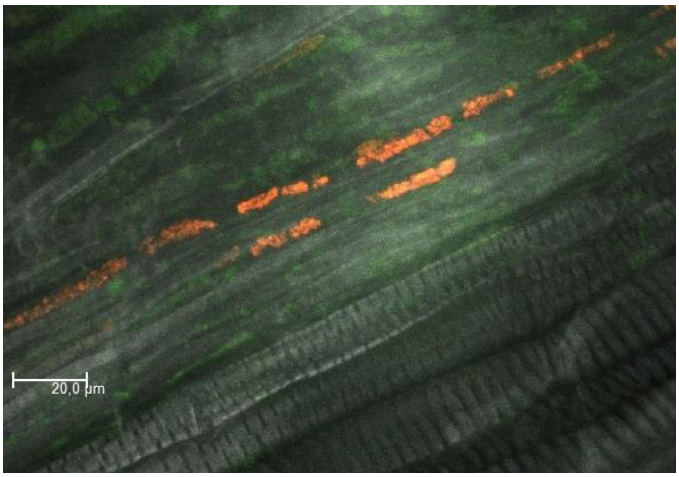

Figure 25: Taxonomically not further characterized *Eubacteria* (red) on fermented leaf samples of *Matricaria chamomilla* L. visualized with the confocal laser scanning microscope.

In the fermented samples of *C. officinalis* L. from Sekem, *Eubacteria* between the system of vascular strands could be detected with the confocal laser scanning microscope (Figure 24). Bacteria thereby were not forming colonies but were evenly distributed over the whole sample. A complete different picture was observed in the fermented plant samples of *M. chamomilla* L. (Figure 25). There, bacteria were colonizing leaf-cells in form of microcolonies and not as on *C. officinalis* L. (Figure 24) allocated distinctly. Beside the detection of taxonomically not further characterized *Eubacteria* (red), it was not possible to detect the desired *Gammaproteobacteria* (yellow) on both of the fermented medicinal plant samples *C. officinalis* L. and *M. chamomilla* L. and neither on fermented cabbage. The attempts to visualize LAB, taxonomically related to the *Firmicutes* with the mix of LGC354 FISH probes (for the visualization of *Firmicutes* with low G+C) did not deliver positive results.

Genomic fingerprint analysis of antagonistic strains from different habitats and plants

The genotypic characterisation of the 40 isolates tested for their antagonistic properties showed different band patterns when looking at their electrophoresis gels performance. Band patterns generated with the BOX_A1 primer created fragments with 6 to 20 bands per lane, ranging in the size from ~400 bp up to approximately 5000 bp. The similarity of some band patterns is evident when looking at the UPGMA dendrogram created using the Pearson's correlation similarity coefficient (Figure 29) with the GelCompar II software. Similarities in the gel patterns can be seen for the two Sekem soil samples Sb3-21 (closest database match according to 16S rRNA gene sequencing: *Bacillus subtilis* subsp. *spizizenii* (NR_024931)) and Sb3-24 (closest database match 16S rRNA gene sequencing: *Bacillus subtilis* subsp. *subtilis* (NR_027552)) in Figure 26. The two isolates were grouped together also in the dendrogram (Figure 29) with a similarity of ~90%. A high similarity was also apparent for the two *M. chamomilla* L. isolates Mc3-4 and Mc5-18. Comparing the band patterns of the whole gel, Mc3-4 and Mc5-18 were the isolates with the highest number of bands visible. The three isolates from the endorhiza of *M. chamomilla* L. from replicate 2 (Mc2Re-18, Mc2Re-21 and Mc2Re-9) in Figure 27 showed a similar performance in the gel

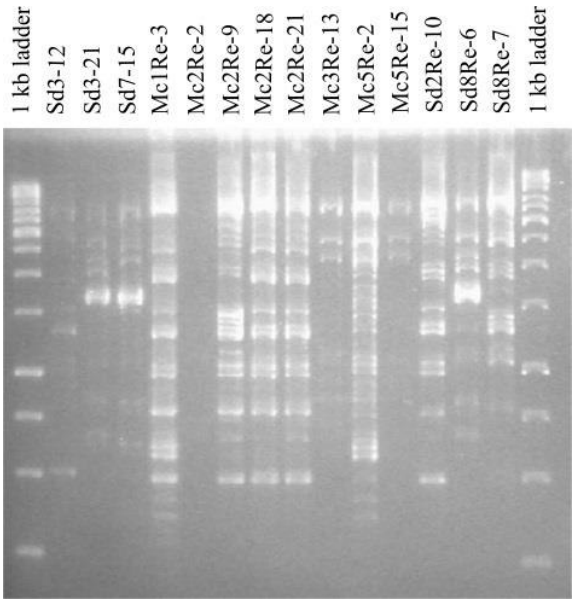

Figure 26: BOX-gel fingerprints of selected bacterial antagonists isolated from different microenvironments.
Isolates were encoded by abbreviations:
(1) soil type or plant species (Wb…desert soil; Sb…Sekem soil, Mc…*Matricaria chamomilla* L., Co…*Calendula officinalis* L., Sd…*Solanum distichum* Schumach. and Thonn.),
(2) replicate (1–4) and
(3) consecutive number of the isolate per replicate.

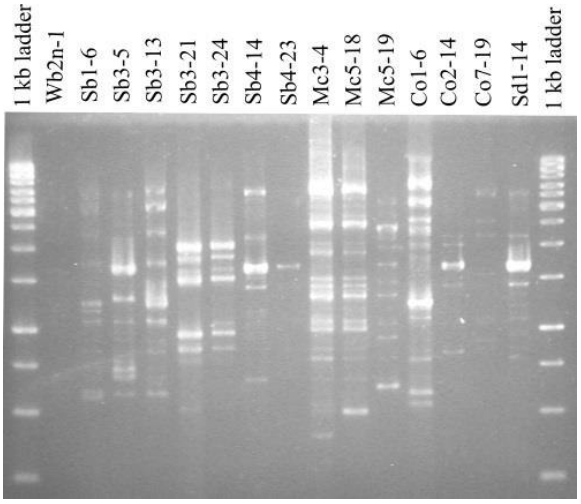

Figure 27: BOX-gel fingerprints of selected bacterial antagonists isolated from different microenvironments.
Isolates were encoded by abbreviations:
(1) Plant species (Mc…*Matricaria chamomilla* L., Co…*Calendula officinalis* L., Sd…*Solanum distichum* Schumach. and Thonn.),
(2) replicate (1–4),
(3) microenvironment (Re…endorhiza, rhizosphere and soil have no further designation), and
(4) consecutive number of the isolate per replicate.

together with the endorhiza sample of Sd2Re-10, an isolate generated from the plant *S. distichum* Schumach. and Thonn.

According to the 16S rRNA gene sequencing, the closest database match was *Bacillus subtilis* subsp. *subtilis* with the accession number NR_027552 for the *M. chamomilla* L. isolates, but *Bacillus mojavensis* (NR_024693) with 100% similarity for the *S. distichum* Schumach. and Thonn. isolate Sd2Re-10. Isolate Sd8Re-7 held also similarity with the former group, showing a similar band pattern but with absent bands in the lower (under 750 bp) area of the gel. Its closest database match was *Bacillus subtilis* subsp. *subtilis*. The isolate Mc1Re-3 from the endorhiza of *M. chamomilla* L. can be regarded as similar, showing a related banding pattern with two thick additional bands at around 600 bp and some weaker bands under the 500 bp band of the standard. Again, *Bacillus subtilis* subsp. *subtilis* was the closest database match for this isolate. Looking at the dendrogram (Figure 29) displaying the results of the band patterns from the defined ARDRA group A this result is again clearly evident. The preceding mentioned isolates were grouped together and do have similarities of over 95%. The previously defined ARDRA group C according to Köberl *et al.* (2013a) containing the two isolates Wb1-13 and Mc4-18 did not show similar performing in the BOX-gel (Figure 28). Wb1-13 showed only 6 distinctive bands all in the upper part of the gel above the 1000 bp band of the standard, while Mc4-18 showed over 10 bands, the smallest one at ~600 bp. According to 16S rRNA sequencing the closest database match for both isolates was *Bacillus endophyticus* (NR_025122) with a similarity of 99%. The band pattern of Mc4-18 though looked identical with the one of Mc2Re-16 (closest database match: *Paenibacillus brasilensis*), an endorhizal sample of *M. chamomilla* L. and with the desert soil sample Sb3-1 (closest database match: *Paenibacillus kribbensis*). From the ARDRA group D outlined by Köberl *et al.* (2013a) containing 8 isolates (Wb2-3, Sb3-1, Mc2-9, Mc5-5, Mc6-4, Mc2Re-16, Mc5Re-14, Sd5Re-24) none of them showed a similar band pattern in the electrophoresis (Figure 28) compared to members of the same ARDRA group.

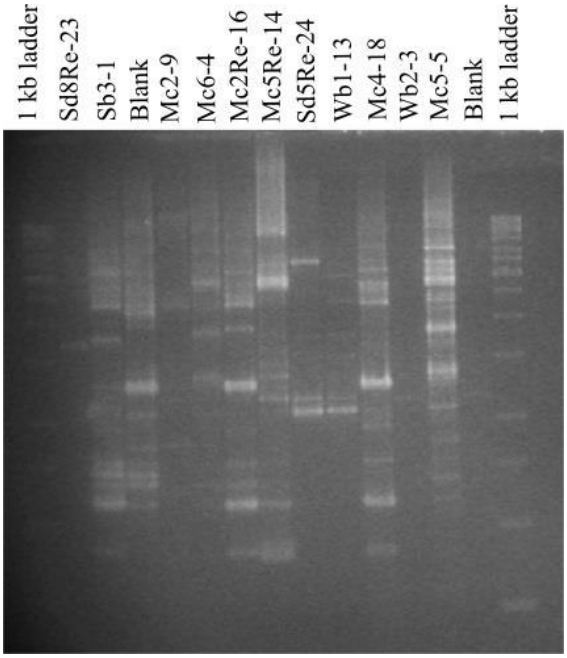

Figure 28: BOX-gel fingerprints of selected bacterial antagonists isolated from different microenvironments.
Isolates were encoded by abbreviations:
(1) soil type or plant species (Wb…desert soil; Sb…Sekem soil, Mc…*Matricaria chamomilla* L., Sd…*Solanum distichum* Schumach. and Thonn.),
(2) replicate (1–4),
(3) microenvironment (Re…endorhiza, rhizosphere and soil have no further designation), and
(4) consecutive number of the isolate per replicate.

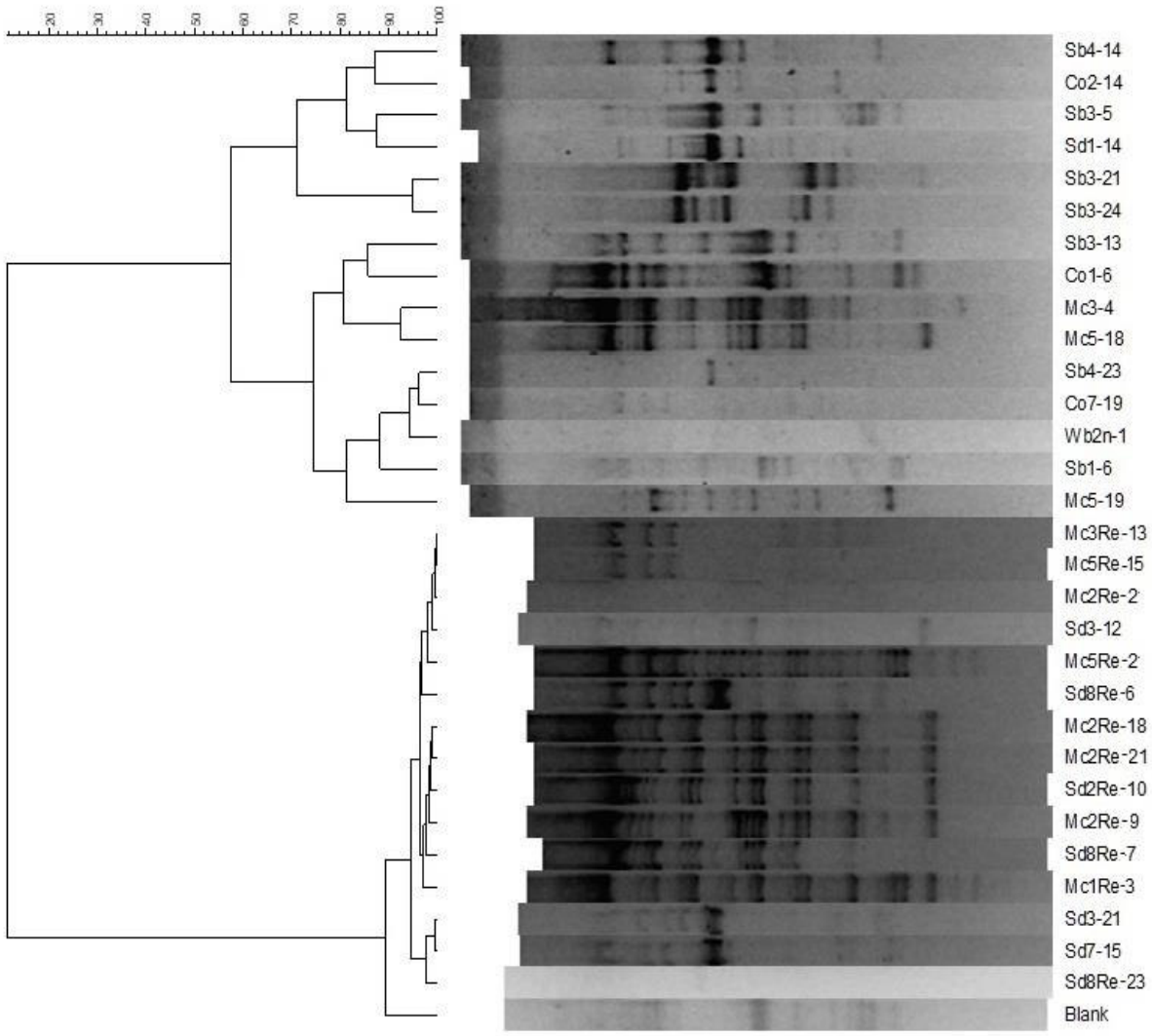

Figure 29: UPGMA dendrogram of isolates from medicinal plants assigned previously as ARDRA group A.
The dendrogram was generated from BOX-PCR fingerprint profiles with the program GelCompar II.
Following settings were used: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); similarity coefficient: curve based: Pearson correlation; position tolerances: optimisation: 4%, position tolerance: 1%.
Isolates were encoded by abbreviations:
(1) soil type or plant species (Wb…desert soil; Sb…Sekem soil, Mc…*Matricaria chamomilla* L., Co…*Calendula officinalis* L., Sd…*Solanum distichum* Schumach. and Thonn.),
(2) replicate (1–4),
(3) microenvironment (Re…endorhiza, rhizosphere and soil have no further designation), and
(4) consecutive number of the isolate per replicate.

Deciphering the genome sequence of plant growth promoting bacteria isolated from agriculturally used farmland and arid desert soil in Egypt

For all three strains, the required concentrations for the sequencing were obtained through pooling two different isolation approaches together (highlighted in grey), from which the sequencing was successfully conducted (Table 14).

Table 14: Performed DNA concentration measurements of selected bacterial antagonists using Nanodrop and PicoGreen. Results of the 230/260/280 ratios were determined to estimate the purity of the different isolations.

Sample/ Isolation date	Concentration with Nanodrop [ng/µl]	260/280	260/230	Concentration with PicoGreen [ng/µl]
Mc5Re-14 11.11.2014	671.2	2.19	2.30	174
Mc5Re-14 26.11.2014	2302.7	1.89	2.07	247
Pool Mc5Re-14 11.11.2014 (30 µl) 26.11.2014 (30 µl)	1757.9	1.94	2.11	211*
Wb2n-11 26.11.2014	626.0	2.00	1.58	220
Wb2n-11 3.12.2014	1632.8	1.92	1.83	251
Pool Wb2n-11 from 26.11.2014 (30 µl) 03.12.2014 (30 µl)	1166.2	1.93	1.75	236*
Co1-6 09.01.2015	1858.4	2.04	2.00	232
Co1-6 27.01.2015	1615.2	1.92	1.97	266
Pool Co1-6 from 09.01.2015 (20 µl) 27.01.2015 (40 µl)	1527.6	1.93	1.82	255*

* **mean value of the two samples before pooling them together.**

Draft genome sequence of Paenibacillus polymyxa strain Mc5Re-14, an antagonistic root endophyte of Matricaria chamomilla L.

Whole-genome shotgun sequencing yielded 136,345 raw reads with 614,329,727 bp of raw sequence. Assembly was completed with the Hierarchical Genome Assembly Process (HGAP) algorithm implemented in PacBio's SMRT Analysis software (Pacific Biosciences, Menlo Park, CA, USA) and subsequently upgraded by PBJelly. The assembly resulted in three contigs summing to 6,038,906 bp, with a maximum contig size of 5,624,359 bp (N_{50} 5,624,359 bp; N_{90} 260,105 bp), a 101.7-fold overall coverage, and a G+C content of 45.23%. The closest relative of Mc5Re-14 based on the full-length 16S rRNA gene sequence is *Paenibacillus polymyxa* strain SC2 (NR_102803: 99% sequence similarity), a plant growth-promoting rhizobacterium with broad-spectrum antimicrobial activity. Digital DNA-DNA hybridization (DDH) using GGDC 2.0 against the genome sequence of *P. polymyxa* strain SC2 (CP002213) estimated a DDH of 86.40 ± 2.44%, indicating that they have 94.41% probability of being the same species and 59.38% probability of being the same subspecies. Annotation was conducted on the RAST web server using RAST gene calling based on

FIGfam version Release70, and additional annotation was completed on the BASys web server using GLIMMER gene prediction. The genome annotation contained 5,433 predicted protein-coding genes, 109 tRNA and 42 rRNA loci, and 450 predicted SEED subsystem features. *Paenibacillus polymyxa* strain Mc5Re-14 genome has broad functional potential and a novel prophage. The genome encodes synthases for mycosubtilin, bacitracin, tyrocidine, gramicidin, and plipastatin, as well as seven additional polyketide synthases. Mc5Re-14 revealed genes for chitinase A1 and extracellular glucanases, and biosynthesis gene clusters for auxin and spermidine production. The prophage encoded by the Mc5Re-14 genome shares homology to phage structural proteins from the giant *Bacillus* siphophage vB_BanS-Tsamsa in *Bacillus anthracis*. The Mc5Re-14 genome revealed promising biocontrol and plant growth promotion capabilities. This whole-genome shotgun project has been deposited in the European Nucleotide Archive (www.ebi.ac.uk/ena) under the accession no. CVPD00000000 (Köberl *et al*., 2015a).

Complete genome sequence of Bacillus amyloliquefaciens strain Co1-6, a plant growth-promoting rhizobacterium of Calendula officinalis L.

Whole-genome shotgun sequencing yielded 245,374 raw reads with 1,486,144,876 bp of raw sequence. Assembly was completed with the Hierarchical Genome Assembly Process (HGAP) algorithm implemented in PacBio's SMRT Analysis software (Pacific Biosciences, Menlo Park, CA, USA) and resulted in a single circular chromosome of 3,922,431 bp, with a 378.9-fold overall coverage, and a G+C content of 46.85%. The closest relative of Co1-6 based on the full-length 16S rRNA gene sequence is *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 (NR_075005: 99% sequence similarity). FZB42 is a well-known PGPR providing the basis of an already commercially available product (Rhizovital® 42, ABiTEP GmbH, Berlin, Germany) with abilities to stimulate plant growth and suppress plant pathogens. Digital DNA-DNA hybridization (DDH) using GGDC 2.0 against the genome sequence of FZB42 (NC_009725) estimated a DDH of 80.30 ± 2.77%, indicating that they have 90.8% probability of being the same species, but only 48.3% probability of being the same subspecies. Annotation was conducted on the RAST web server using RAST gene calling based on FIGfam version Release70, and additional annotation was completed on the BASys web server using GLIMMER gene prediction. The genome annotation contained 3,913 predicted protein-coding genes, 86 tRNA and 19 rRNA loci, and 457 predicted SEED subsystem features. The genome encodes synthases for mycosubtilin, plipastatin, and

surfactin antibiotics, which most probably contribute to Co1-6's promising abilities for pathogen suppression. Co1-6 revealed six additional polyketide synthases, some in up to seven copies, and a dimodular nonribosomal peptide synthase. We further identified genes most probably involved in direct promotion of plant growth, such as biosynthesis gene clusters for rhizobactin siderophores, spermidine, and auxin. This whole-genome shotgun project has been deposited in the European Nucleotide Archive (www.ebi.ac.uk/ena) under the accession no. CVPA00000000 (Köberl *et al.*, 2015b).

Draft Genome Sequence of Streptomyces sp. strain Wb2n-11, a desert isolate with broad-spectrum antagonism against soil-borne phytopathogens

Whole-genome shotgun sequencing yielded 123,881 raw reads with 604,678,994 bp of raw sequence. Assembly was completed with the Hierarchical Genome Assembly Process (HGAP) algorithm implemented in PacBio's SMRT Analysis software (Pacific Biosciences, Menlo Park, CA, USA) and subsequently upgraded by PBJelly. The assembly resulted in five contigs summing to 8,228,099 bp, with a maximum contig size of 7,583,077 bp (N_{50} 7,583,077 bp; N_{90} 284,642 bp), a 73.5-fold overall coverage, and a G+C content of 71.03%. The closest relatives based on the full-length 16S rRNA gene sequence are *Streptomyces scopiformis* strain A25 (NR_114403) and *Streptomyces enissocaesilis* strain NBRC 100763 (NR_041411), both with 99% sequence similarity. Whole-genome alignment using Mauve revealed conserved regions between Wb2n-11, *Streptomyces griseus* subsp. *griseus* strain NBRC 13350 (NC_010572), and *Streptomyces avermitilis* strain MA-4680 (NC_003155), the closest available reference genomes. However, digital DNA-DNA hybridization using GGDC 2.0 against these two genomes excluded the probability of belonging to one of these species. Annotation was conducted on the RAST web server using RAST gene calling based on FIGfam version Release70, and additional annotation was completed on the BASys web server using GLIMMER gene prediction. The genome annotation contained 7,643 predicted protein-coding genes, 65 tRNA and 21 rRNA loci, and 434 predicted SEED subsystem features. Wb2n-11 revealed several genes which could contribute to direct and indirect plant growth promotion. We identified genes putatively involved in the biosynthesis of a broad spectrum of antibiotics, such as synthases for bacitracin, tyrocidine, linear and cyclic gramicidin, erythronolide, surfactin, and synthetases for triostin. The genome revealed 14 additional polyketide synthases, some in up to ten copies, and three copies of a dimodular nonribosomal peptide synthase for syntheses of complex secondary metabolites. Wb2n-11

further encodes the production of extracellular cell wall degrading enzymes (chitinase C, extracellular proteases and glucanases), siderophores, auxin, and spermidine. Wb2n-11 has a complete ectoine biosynthesis and regulation gene cluster that contributes to its survivability under extreme conditions. This whole-genome shotgun project has been deposited in the European Nucleotide Archive (www.ebi.ac.uk/ena) under the accession no. CVPB00000000 (Köberl *et al*., 2015c).

Discussion

Fundamentals of lactic acid bacteria and their potential as natural and safe source for industrial applications

Lactic acid bacteria and their by-products (e.g. bacteriocins) are already applied industrially in the control of foodborne pathogens (Chikindas *et al.*, 2018) and looking towards the future they will be even more implicated in the food and agricultural sector as well as in the pharmaceutical field. They possess high potential as probiotics positively influencing human health and protecting the natural gastrointestinal flora from harmful pathogens. Additionally, combating against plant pathogens on agricultural commodities, thereby leading to savings of chemical fertilizers are not only visions, but already partly implemented concepts. As the field of probiotics is becoming more and more popular, the isolation as well as the more defined characterization of strains belonging to the *Lactobacillales* is of eminent importance. The potential of plant-inhabiting LAB in the preservation of foods and feeds due to their ability of production of antibacterial and antifungal compounds (Magnusson & Schnürer, 2001; Magnusson *et al.*, 2003; Sjögren *et al.*, 2003; Corsetti *et al.*, 2004) is known since centuries and has been demonstrated by many studies (Gajbhiye & Kapadnis, 2016).

During the course of this thesis, one aspect was the enrichment of lactic acid bacteria through the performance of a fermentation of different plant material and the evidence of the presence of potentially probiotic strains on medicinal plants. Key issues near the isolation and cultivation of probiotic bacteria was also the verification of the viability of the strains and the documentation of their positive effect regarding human health and disease prevention. In the presented study, LAB with leaf ecto- and endophytic origin from *Asteraceae* medicinal plants were detected and isolated, revealing an impressive level of plant-specificity. LAB have previously been ascertained as natural inhabitants of the ectospheric phyllosphere of a broad spectrum of plants, whereby remarkable presence was uncovered in the leaf microbiomes of plants exposed to harsh environmental conditions and grown under organic management, respectively (Ott *et al.*, 2001; Zwielehner *et al.*, 2008; Vokou *et al.*, 2012) – both true for our sampling sites. An endophytic lifestyle of LAB has been primarily investigated for cereal crops, in their processing they serve as natural inoculum in sourdough fermentation (Gaggìa *et al.*, 2013; Minervini *et al.*, 2015). LAB have also been detected in quite high abundances in

the endosphere of Mediterranean olive trees, revealing significantly higher presence in eastern than in western Mediterranean regions (Müller *et al*., 2015). Recently, a variety of endophytic LAB has also been detected and cultivated from *Cucurbitaceae* seeds, suggesting edible cucurbit seeds as probiotic food product (Khalaf *et al*., 2016). However, to our knowledge, medicinal plants have never been investigated in this respect, although a combination of their phytotherapeutic metabolites with a probiotically active microbiome following fermentation could bear a beneficial health effect in two different ways in parallel. Aside their function as probiotics and the promotion of human health, LAB also play a role in agriculture by promoting plant health through their antimicrobial potential against several plant pathogens (Trias *et al*., 2008; Shrestha *et al*., 2014). The disadvantage of chemical means which are considered to be toxic and the fact that some of them cannot be used for postharvest treatment in agriculture, the rising resistance of some pathogens against the chemicals and also the high costs of the development of new chemicals have led to a higher acceptance of microorganism-based treatment methods (Berg, 2009).

The majority of LAB are classified as having GRAS (generally-recognized-as-safe) status and their application is quite safe, both from human and environmental point of view (Salminen *et al*., 1998; O'Sullivan *et al*., 2002). However, there are also exceptions, for instance some species of *Enterococcus* or *Streptococcus* can cause opportunistic infections in humans, whereby the intrinsically resistance of LAB to many antibiotics is an additional risk factor (Reid *et al*., 2001; Lindenstrauss *et al*., 2011; Jans *et al*., 2015). In a recently published WHO list of priority pathogens for R&D of new antibiotics, *Enterococcus faecium* was listed with high and *Streptococcus pneumoniae* with medium priority:
http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en.
The genus *Lactobacillus* is most emphasized in literature for its beneficial activities, as its isolation is reported from a vast diversity of fermented and unfermented sources. However, it was reported that members of the genera *Lactococcus*, *Enterococcus*, *Pediococcus* and *Leuconostoc* have at least equal potential (Gajbhiye & Kapadnis, 2016). Under careful consideration of the safety and antimicrobial spectrum of LAB, they bear the potential for an excellent alternative to synthetic chemical pesticides in sustainable agriculture, potentially even combined with a probiotic effect on human and animal consumers.

The (lactic acid bacterial) plant microbiome – origin of plant health and biocontrol

In the course of the implemented fermentation, natural phyllospheric LAB were successfully enriched and different species of *Lactobacillus* and *Enterococcus* could be cultivated from the fermented medicinal plants. Besides the leaf surfaces, also the leaf endosphere could be identified as source for biopreservative LAB. The two investigated field-grown *Asteraceae* medicinal plants revealed a plant-specific natural leaf microbiome, although they were grown in direct proximity to one another. Hence, different abiotic factors can be excluded as the reason for the differences in microbial composition. It can therefore be concluded that each plant provides a habitat for a varying microbiome and enriches a specific bacterial composition. This phenomenon was already shown by several studies before, but mainly focusing on the rhizosphere and root microbiome (Berg *et al*., 2009, Hartmann *et al*., 2009, Bulgarelli *et al*., 2012, Bulgarelli *et al*., 2015), while in the presented study this could be confirmed for the phyllosphere surface.

The presence of probiotic lactic acid bacteria on medicinal plants opens a new field where further investigations should be undertaken to elucidate their role in plant health and possibly the plant's medicinal properties. Although no antagonistic strains against the tested soil-borne phytopathogens *Fusarium culmorum* E1, *Verticillium dahliae* V25 and *Rhizoctonia solani* AG4 were found in this study, it cannot be excluded, that they perhaps still possess a positive effect on plant productivity. As the plant health promoting effect of lactic acid bacteria through their antimicrobial potential against several plant pathogens was already shown in other studies (Trias *et al*., 2008; Shrestha *et al*., 2014), culture assays should be repeated under modified conditions. Moreover, strains should be tested *ad planta* in their natural habitat and not just *in vitro*, excluding effects of growth medium and nutrient supply. Further investigations in this field are promising, as several studies have shown that each plant harbors their own microbiome (Berg *et al*., 2009, Hartmann *et al*., 2009, Bulgarelli *et al*., 2012, Bulgarelli *et al*., 2015) and that the microbiome is responsible – to at least some extend – for the improvement of plant health and quality. It was, for instance, demonstrated that the two furanoids 2,5-dimethyl-4-hydroxy-2H-furanone (DMHF) and 2,5-dimethyl-4-methoxy-2H-furanone known as flavor compounds in strawberries could be enhanced by sprinkling strawberry plants with methylotrophic bacteria (*Methylobacterium extorquens*) during the flowering stage (Verginer *et al*., 2010). Especially the phyllosphere is an auspicious place to

look for microorganisms as it provides a huge area with an estimated surface of 1,017,260,200 km^2 (upper and lower leaf surface), said to be twice as great as the land surface (Vorholt *et al.*, 2012). Living conditions for organisms on the leaf surface may be difficult to handle due to environmental determinants involving climatic conditions like UV radiation, temperature and moisture fluctuations, wind or heavy rainfall. Moreover, less nutrients are available in the phyllosphere compared to the rhizosphere. Despite those harsh conditions, it is assumed that up to 10^7 microbes per cm^2 colonize the leaf surface, thereby showing colonization patterns varying within the leaf surface depending on the leaf structures like veins, hairs and stomata (Turner *et al.*, 2013). Assessing this quite rigid environment, one ends wondering where the microorganisms on the plant's surface come from. Microorganisms found on the surface of plants can be derived into two groups of leaf colonizers named *ad hoc* and local reservoirs. *Ad hoc* dispersal includes colonizers introduced through the air, water droplets, dust particles but also microorganisms allocated through insects. Local reservoirs involve the transmission through the nearby environment including soil and other plant matter as well as pollen (Vorholt *et al.*, 2012). The phyllosphere (term invented 1955 by F. T. Last) is besides the rhizosphere a habitat for microorganisms encouraging the health maintenance of agricultural but also non-agricultural plants and therefore harbors significant potential in the field of biocontrol and plant growth promotion (Aleklett *et al.*, 2014). According to Berg (2009), plant growth promotion can be achieved directly through the interaction of the microbes with the plant but also indirectly through antagonistic activity of the microbes against plant pathogens. These natural interactions harbor a plethora of advantages over conventional pesticides and fertilizers. Microbes are effective in small quantities, decompose more quickly and do have a reduced environmental damage compared to conventional pesticides. Besides, microbes are safer and possess a smaller risk in affecting human health. Compared to chemical/synthesized pesticides and fertilizers they show an enhanced targeted activity and multiply themselves, though their reproduction is controlled by the plant and indigenous microbial populations. Additionally, as there are lots of resistance mechanisms, resistance development is minimized (Berg, 2009).

Fermentation: an overview of the enrichment and cultivation of lactic acid bacteria

Regarding the cultivation of the potential LAB, the picking of diverse bacteria from all the approaches was in respect difficult due to only optical differences were consulted as distinctive characteristics. Therefore, it is likely that through the discrimination of only morphological differences, colonies of the same bacterium were picked and streaked out manifold for cultivation and later analyses. Attempts to exclude bacteria not belonging to LAB was done through the implementation of the Katalase test. The H_2O_2 decomposition in the presence of the enzyme Katalase induces the formation of bubbles, which can be observed. As all lactic acid bacteria lack the enzyme Katalase, only isolates which were negative in the test were selected for further analysis. The KOH test was done as a quick test for analyzing the Gram staining of the isolates. The cell wall of Gram-negative bacteria is lysed in the presence of the base resulting in the release of DNA along with raising the viscosity of the bacteria-KOH suspension. The operability of the KOH test was first analyzed with a known Gram-negative test strain which was already hard to differentiate from a known Gram-positive test strain. Due to the practicability of this, outcomes of the Katalase test were weighted higher due to it delivered unambiguous results.

All the cultivation approaches were performed on agar plates enriched with Cycloheximide to prevent growth of contaminating fungi, which could have affected the growth of the desired bacteria as well. Concerning the CFUs of the different cultivation approaches (aerobically, anaerobically, MRS vs. R2A agar) the highest CFU value was observed on the plates cultivated aerobically on R2A agar – a result, which was expected like this. The low nutrient R2A agar known to prevent the expansion of fast growing bacteria is generally used for the cultivation of heterotrophic, slow growing bacteria (Reasoner & Geldreich, 1985). MRS agar is a medium developed from De Man, Rogosa and Sharpe specifically for the cultivation of lactobacilli capable to substitute the previously used medium containing tomato juice, which is difficult to handle due to its variability and the laborious preparation. The inclusion of the surfactant Tween 80 citrate and acetate to the medium resulted in improved growth of lactobacilli when added to the formula. Besides magnesium also manganese was added to the medium, whose demand is increased in the presence of citrate. With those considerations, the lactobacilli grew much better on the newly developed medium than on the reference medium containing tomato juice (De Man *et al.*, 1960). At low pH, the added ammonium citrate acts

as a selective agent through growth inhibition of a lot of microorganisms, like streptococci and molds (according to the product datasheet of Carl Roth). This circumstance explains why the $\log_{10}$ CFU ml^{-1} for the cultivation approaches on R2A was on average higher compared to the MRS agar. Colonies grown on R2A agar appeared also earlier than on the MRS agar due to the selectivity of the MRS as a result of the lower pH for the rather slow growing lactobacilli. Moreover, as expected, the average $\log_{10}$ CFU ml^{-1} of the aerobically cultivated approach was higher (7.41) than the one treated anaerobically (7.02) and microaerophilically (7.09). Mainly responsible therefore is the fact that through aerobic respiration a higher ATP yield allows faster reproduction and a selection pressure can be build up in growing cultures under anaerobic conditions (Hofkin, 2017). The CFUs were not significantly lower or higher for the approach of the non-acidic calendula approach compared to the other approaches with lower pH. Though, a notable fact was the odor. While the sauerkraut had its typical sour, acidic smell, the fermentation approaches of the chamomile had an almost "fine scent" comparable with hay, whereas the marigold approaches had an unpleasant odor of something rotting (eggs?). It seemed that a so called "faulty fermentation" occurred, where not the lactic acid bacteria took predominance, but other microorganisms and molds.

In general, it should be mentioned that the fermentation was not done in a sterile environment. The preparation (chopping) of the starting material as well as the fermentation of the approaches in the glass jars was not carried out under aseptic conditions, so an intrusion of "external germs" cannot be excluded. Another source of error is the withdrawal of samples which was done every week during the six-week ongoing fermentation. This disturbance procedure may have influenced the equilibrium of the fermentation as 15 ml of the fermentation broth were withdrawn and refilled with 0.85% NaCl so that the approaches were again covered with liquid.

In vitro antagonistic potential of isolated lactic acid bacteria towards plant and human pathogens

Regarding the antagonistic potential of the isolated lactic acid bacteria, an antagonistic activity towards plant or human pathogens could not be examined for the isolates. One reason might be the growth advantage of the fungi and human pathogens against the slow growing lactic acid bacteria. The MRS agar which was used in all cultivation experiments was not

suitable for the implementation of the antagonistic tests and had to be surrogated by Waksman and TSB agar. The added ammonium citrate in the MRS bouillon acts as a selective agent through growth inhibition of a lot of microorganisms like streptococci. For example, *Staphylococcus aureus* ATCC 25923 is inhibited from growth according to the product datasheet of MRS bouillon from Carl Roth and it can also inhibit molds growth. In the study of Stiles *et al*. (2002) the added sodium acetate from the MRS bouillon showed that *Fusarium* (particularly *F. culmorum*) was quite sensitive against it, responding with strong growth inhibition in its presence. Furthermore, synergistic effects were observed when media enriched with sodium acetate in combination with the lactic acid bacterium were combined (Stiles *et al*., 2002). Although pretests regarding the growth of the potential LAB on Waksman and TSB agar were done, the growth conditions were not optimal as in the MRS media some additives (e.g. surfactant Tween 80 citrate and acetate) are added to promote the growth of LAB. To bypass the growth advantage of the fungi/bacteria, the incubation of LAB prior to the addition of the testing fungi/bacteria as it was done by Magnusson & Schnürer (2001) would have been beneficial. They incubated *L. coryniformis* as two 2-cm-long lines on MRS agar plates for 48 h before overlaying them with a layer of inoculated fungal/bacterial malt extract soft agar. The plates were again incubated for 48 h before measurement of the inhibition zone (Magnusson & Schnürer, 2001). Two other methods to test the antagonistic potential were also described by Magnusson & Schnürer (2001). They describe an agar well diffusion assay and a microtiter plate well assay to test the antagonistic potential. Although, the previously mentioned inhibition effect of sodium acetate was not considered in this paper, and all three mentioned methods were performed on MRS media. Therefore, a combination of exchanging the media into an appropriate (good/acceptable growth for LAB accompanied by an incubation of LAB prior to addition of the testing microorganisms) should be considered. Taking the numerous amount of publications describing the antagonistic properties of LAB into account (e.g. Magnusson & Schnürer, 2001; Stiles *et al*., 2002; Trias *et al.*, 2008; Darsanaki *et al.*, 2012; Shokryazdan *et al.*, 2014), the antagonistic test with the isolated potential LAB of this project should be repeated with conditions appropriate for the LAB to elicit their antagonistic potential.

Characterization of isolated lactic acid bacteria from various plant sources using ARDRA and rep-PCR

To introduce a little order in the high number of isolates, the method of ARDRA was used to group similar strains. Screening the 394 potential isolates based on their individual ARDRA performance together with the sequencing of selected isolates allowed the classification into five taxonomically distinctive groups. All five groups could be confirmed belonging to the order of *Lactobacillales* through sequence analysis of randomly picked isolates covering all five ARDRA groups. Sequencing revealed *Lactobacillus* as predominant genus in four of the five ARDRA groups, determining *L. fabifermentans, L. pentosus*, *L. coryniformis, L. brevis, L. casei, L. paracasei and L. nenjiangensis* as apparently prevailing species. The sequenced isolates of the fifth ARDRA group belonged to the genus *Enterococcus* with *Enterococcus casseliflavus* as the closest database match after BLAST comparison analysis. As all three isolates sequenced from the ARDRA group D belonged to the genus *Enterococcus*, it can be hypothesized that the 173 isolates of the big ARDRA group D could be classified as enterococci. This also fits together when looking at the results of the amplicon sequencing, where the *Enterococcaceae* where one of the most abundant families belonging to the class of *Bacilli*. The genus *Enterococcus* found on fresh fruits and vegetables is often associated with human fecal contamination, especially the species *E. faecalis* and *E. faecium*, whereas *E. casseliflavus* and *E. mundtii* are more prevalent in the environment (Al-Kharousi *et al.*, 2016). Although enterococci are often ranked as spoiling contaminants, studies have brought them in connection as important species in the ripening process of Mediterranean cheese influencing their characteristically taste and flavor. Besides, enterococci were not only found in cheese, but also in fermented olives and other vegetables and are used as probiotic starter cultures. However even though they are used as probiotics, enterococci have been related to a number of human infections (e.g. infections of the urinary tract and central nervous system as well as intra-abdominal and pelvic infections), making them a controversial group of probiotic lactic acid bacteria. Apprehensions regarding their possibility to transfer resistance genes and genes encoding virulence factors to other bacteria and additional the fact, that enterococci are among the most common known nosocomial pathogens call their use as probiotics into question. On the other hand, their health promoting competence in conjunction with their activity against other spoilage and pathogenic bacteria (e.g. *Listeria*) due to their production of potent bacteriocins make them potential candidates as cultures in the food production (Foulquié Moreno *et al.*, 2006). Although contamination from the "farm to the fork" (Al-

Kharousi *et al*., 2016), meaning contaminations do not only occur at the planting location itself but also throughout transportation, package, etc., Berg *et al*. (2014) found that these opportunistic bacteria do also lead a live as endophytes in the plants endosphere and in the rhizosphere. With the increased consumption of raw vegetables harboring those opportunistic bacteria in connection with the rising number of immunocompromised individuals consuming them, it is not remarkable, that food-borne infections related to the consumption of contaminated vegetables are in continuous upswing (Berg *et al*., 2014). In the study of Al-Kharousi *et al*. (2016), the highest $\log \mathrm{CFU\,g^{-1}}$ for *Enterococcus* was found on Omanian cabbage, whereas the group D in this present study from which all *Enterococcus* isolates were derived, were mainly gathered from plant material of the medicinal plants except for one sample which was derived from fermented cabbage. Other closest database matches regarding the sequencing analysis in this study however were all members of the genus *Lactobacillus*, among them *L. pentosus*, *L. plantarum*, *L. brevis* and *L. casei*, all of them well known in the use as probiotics. In general, lactic acid bacteria are not only interesting due to their probiotic properties but are, due to their production of bacteriocins, effective and promising candidates as preserving agents and also as source for future antibiotics against multiple drug resistant pathogens. Their use in the food sector is advantageous over bacteriocins derived from other microorganism due to their long history of use back to ancient times. Lactic acid bacteria and their by-products are Generally Regarded as Safe (GRAS) as a human food ingredient by the U.S. Food and Drug Administration (FDA) and do possess other advantages like a high thermal stability and wide active pH range. What even makes them more interesting for the food industry are their colorless, odorless, and tasteless properties. In general, bacteriocins are antimicrobial peptides synthesized in the ribosomes. Their action as food preservative can be implemented indirectly by applying bacteriocins in their purified form or by directly inoculating food with bacteriocin-producing bacteria (e.g. LAB). Research is also undertaken in deploying bacteriocins in the packaging film or surface of the food. Regarding their pharmaceutical properties, the purpose has been to enhance the specific activity of certain bacteriocins against clinically relevant (multiresistant) pathogens as well as an enhanced bioactivity and a higher stability within the body. An attempt is also the reduction of negative effects on the natural gut microflora. Promising in this field is the amenability of bacteriocins to bioengineering due to their relative simple biosynthetic mechanism compared with conventional antibiotics (Perez *et al*., 2014).

A subsequent attempt used in this study to remove duplicates was the method of rep-PCR fingerprinting, another cultivation-dependent method applied to order the isolates based on their individual BOX-gel patterns. However, the distinguishable band patterns looked all very similar to each other, preventing them from being classified regarding their performance in the electrophoresis. The rep-PCR fingerprinting used for the ability to characterize different LAB was already implemented by Gevers *et al*. (2001). In their study, the rep-PCR oligonucleotide primer BOXA1R (5PCTACGGCAAGGCGACGCTGACG-3P), the primer pair REP1R-I (5P-IIIICGICGICATCIGGC-3P) and REP2-I (5P-IIICGNCGNCATCNGGC-3P) and the $(GTG)_5$ (5P-GTGGTGGTGGTGGTG-3P) primer were tested. The BOXA1R resulted in a banding pattern containing only 0-6 visualized PCR products, electrophoresis with REP1R-I and REP2-I showed fingerprints of 1-10 bands in contrast to 10-20 visible bands with the $(GTG)_5$. With the preferable $(GTG)_5$ primer they managed to group the tested reference strains in separate clusters regarding their taxonomic affiliation. As it seems, the $(GTG)_5$ primer would have been a better choice to taxonomically distinguish the potential LAB isolates of our study as only around six bands were visible on the gels, all looking rather similar making a differentiation not feasible. Other methods for studying LAB like protein profiling, 16S rRNA sequencing, ribotyping, and pulsed-field gel electrophoresis (PFGE) mentioned in the publication of Gravers *et al*. (2001) are according to the authors either limited in their resolving power, are too laborious or require a species-specific methodology.

Cultivation-independent characterization: analysis of the bacterial colonization of native and fermented plant material

Based on the results of the 16S rRNA gene amplicon sequencing, the endosphere of both medicinal plants was heavily dominated by *Gammaproteobacteria*, as it was already described for some other endophytic plant communities, e.g. in tomato, lettuce and banana plants (Jackson *et al*., 2013, Romero *et al*., 2014, Köberl *et al*., 2015d). *Gammaproteobacteria* were also identified as the most prevalent bacterial class in both medicinal plant ectospheres, making up approximately 50% of the total bacterial colonization. The class of *Bacilli* to which the LAB are belonging were found in a much lower abundance in the original phyllosphere microbiome, but *Lactobacillales* were enriched during the decomposition process of plant material. In the course of the fermentation, *Gammaproteobacteria* were supplanted by communities belonging to the phylum of

Firmicutes, besides *Lactobacillales* also *Clostridiales*. Among the LAB, *Enterococcaceae* were identified as the most dominant family in both plants. The remaining LAB community was remarkably different between the fermentation approaches of the two medicinal plants: *M. chamomilla* L. revealed higher relative abundances of *Lactobacillaceae* and *Carnobacteriaceae*, while *C. officinalis* L. showed higher presence of *Leuconostocaceae* and *Streptococcaceae*. Interestingly, the most abundant family belonging to the *Bacilli* were not LAB but *Planococcaceae*. The genera *Planomicrobium* and *Sporosarcina* of the family of *Planococcaceae* have previously been detected as inhabitants of native Egyptian desert soil (Köberl *et al*., 2013c). However, in the phyllosphere microbiome of the investigated desert farm plants primarily *Lysinibacillus* and *Planococcus* were detected, whereby especially *Lysinibacillus* demonstrated high tolerance to the acidification during the fermentation process (down to a pH of 2.8). The pH certainly had a huge impact on community dynamics within the fermentation approaches in general. While the pH of the chamomile approach after six weeks of fermentation with 2.8 was highly acidic, the marigold fermentation reached only a pH of 6.4.

Another cultivation-independent method applied to characterize the colonization of medicinal plants was SSCP analysis, where single-stranded DNA is created through lambda exonuclease digestion, which after heat denaturation is renaturated and afterwards applied to a nondenaturating gel. The principle behind this method lies in the fact that single-stranded DNA forms secondary structures depending on its nucleotide sequence when administered to nondenaturing conditions. Different conformations of the initial DNA show different operating performance in nondenaturing gels due to altered electrophoretic mobilities (Schwieger & Tebbe, 1998). For the amplification of bacterial DNA, variable regions of the otherwise highly conserved 16S rRNA gene found in all bacteria are used. Through the selection of regions of the gene which are conserved in one taxa but are different in the other, the phylogenetic breadth of the primers can be adjusted. Depending on which region of the small subunit of the rRNA gene is chosen, a differentiation at genus and higher taxonomic levels is possible. If the internal transcribed spacer (ITS), who lies between the small and the large subunit gene is used, discrimination at species and subspecies levels is achievable. (Blackwood *et al*., 2005, Leung & Yip, 2008). During the course of the experiment, it has been noted that the used *Firmicutes*-specific primer didn't cover the desired lactic acid bacteria (Blackwood et al., 2005). Moreover, many attempts failed to amplify the DNA of the different taxonomic groups. Problems occurred in particular for approaches where a nested

PCR had to be carried out. The right amount of target DNA from the first PCR approach to carry out the second PCR was difficult to estimate. A concentration of target DNA too low/high can cause insufficient amplification leading to no visible band on the control gels. A nested PCR approach was performed functioning on the principle of performing a first PCR approach followed by a second PCR approach where material from the first PCR is used as a template, allowing a more defined amplification. This enables the detection of less abundant communities, which are enriched in the course of the first amplification, providing enough starting material for the second PCR approach. The sensitivity is increased up to 1000-fold compared to a conventional PCR approach but requires more effort and is linked to a higher contamination risk (Kalland, 2009). Amplification of DNA taken from PCR approaches which showed no band on the gel from the first PCR sometimes was successful as the amplified amount of DNA was too low to be captured in the electrophoresis but was high enough and sufficient to be amplified in the second PCR approach. For some samples it was – also after many attempts undertaken – not possible to get amounts of DNA high enough to carry out a SSCP analysis. Amplification of lactobacilli from native cabbage resulted in only a few very weak bands on the gels of the lactobacilli-specific PCR, although for nearly all samples a band was visible on the gels after performing the first unspecific PCR of the nested PCR approach. Also, amplification of lactobacilli from the phyllosphere of the medicinal plants was rather modest as amplification worked not well for those sample approaches either. Facing those problems in the amplification to gather enough DNA for applying to the SSCP gels, no data regarding the SSCP fingerprints of the genus *Lactobacillus* of the native/fermented plant samples could be investigated.

Beside the problems which occurred in the amplification of the template DNA for the SSCP gels, also reamplification of the bands cut out from the SSCP gels did not generate satisfying results. One hypothesis trying to explain this aspect is that one band from the gel contains only small amounts of DNA, the circumstance that some bands were also very thin, making it very difficult to cut them out and re-extract DNA probably. For the bands, where reamplification worked, the NCBI BLAST comparison was sobering as no significant similarity could be found for most of the samples when running the algorithm over the gathered sequences. For four samples from approaches of fresh cabbage, an alignment was achieved (all from the total fungal community SSCP gel) and *Wallemia ichthyophaga* was the closest database match. It seems not very likely that this fungus, known so far as the most halophilic fungus on earth, was really present in the samples in high amounts. Its natural

habitats are high saline solutions in e.g. salterns. It was found that *Wallemia ichthyophaga* is obligate halophilic, not growing under salinities under 10%, but able to thrive up to saturated NaCl solutions (Zajc *et al*., 2014). Such high salt concentrations are not found on fresh cabbage samples and were neither achieved even in the fermentation process, where only 2% (w/w) NaCl was added. The match of *Fomitiporia mediterranea* is rather unlikely due to it is a fungus from the phylum of the *Basidiomycota* and the sequenced band was extracterd from the gel targeting the *Ascomycota*. *Bipolaris sorokiniana* from the phylum *Ascomycota*, which matched two reamplified samples is known as one of the most serious foliar diseases in wheat and barley leading to significant yield losses (Kumar *et al*., 2002). It was already shown 1993, that toxic metabolites of *Bipolaris sorokiniana* produced wilting and chlorosis symptoms on *M. chamomilla* L. (Raghuchander *et al*., 1993). The ascomycete *Setosphaeria turcica*, was found in the reamplified bands from two different SSCP gels (one in the total fungal community, the other in the *Ascomycota* community). It is the causer of the Northern corn leaf blight (NCLB) in maize (Galiano-Carneiro *et al*., 2017). With the exception of *Wallemia ichthyophaga,* all NCBI blast matches were connected with fungi, which are associated with plant diseases. This may be due to a lot of research is towards plant disease control in conjunction with sequencing the genomes to find a missing puzzle piece in combating yield losses caused by those pathogens. This again underlines the importance of plant disease control and research in the field of biocontrol.

In situ visualisation of bacterial inhabitants on native and fermented plant samples

The *in situ* visualisation of bacterial inhabitants on native and fermented plant samples regarding different bacterial communities was implemented with the method of confocal laser scanning microscopy in combination with fluorescence *in situ* hybridization. The colonization patterns visible under the microscope on the different parts of the plants and for the different bacterial taxa however should be considered cautiously since the colonization on all plants found in this study was rather rare. Generally, it should be taken into account that for all tested probes not enough replicates for meaningful analysis were acquired showing the same colonization patters. When analyzing the rhizosphere colonization of lettuce with *Streptomyces*, Chen *et al*. (2016) found a distribution of bacteria looking quite similar to the distribution represented in Figure 22. In their study the analyzed *Streptomyces* was growing

filamentous on the surface as well as on the main and lateral roots, as well as on and in the proximity of root hairs. The mycelium structure found on *M. chamomilla* L. therefor could be an indication to be from the family of *Streptomycetaceae*. In the study of Köberl *et al.* (2013a), antagonistic isolates of *Streptomyces* were found only in the desert soil of Sinai but not in the rhizosphere of the medicinal plants on agriculture land. Looking at the results of the amplicon sequencing of the fresh medicinal plant samples of this study the *Actinobacteria* though, to which the *Streptomyces* are related, were one of the major taxonomic classes found in the ectosphere of both medicinal plants. The colonization of *Gammaproteobacteria* on pollen as it was shown for blossoms of *M. chamomilla* L. in Figure 23 was already shown on pollen of pumpkin by Fürnkranz *et al.* (2012). As described there, pollen grains therefore are not only important for the plant's fertilization but hold the function as a potential bacterial vector between flowers too.

Although a low amount of *Gammaproteobacteria* could be detected with the microscope, the CLSM images do not meet their actual colorization frequency due to the fact that *Gammaproteobacteria* beside *Alphaproteobacteria*, the *Bacilli* and the *Actinobacteria* were among the major taxonomic classes located on the unfermented plant material. Despite several attempts were undertaken in the visualization of lactic acid bacteria on the unfermented and fermented plant samples, no lactic acid bacteria could be detected through the application of FISH-CLSM. In the microscopic detection of them, two fluorescence probes were used. One of them binding unspecific to all *Eubacteria*, the other one for *Firmicutes* (LGC354A/B/C) binding to the *Bacilli* sub-branch of Gram-positive bacteria with a low DNA G+C content (orders *Bacillales* and *Lactobacillales*), as it was indicated by Meier *et al.* (1999). Bacteria in the overlap image colored in the mix of the two colors, in this case yellow (as a result of the red *Eubacteria* and the green *Firmicutes*-specific probe) could be defined with high probability as *Firmicutes* (with low G+C content). Performing this method, unspecific bindings of the probes can be more or less excluded due to it is very unlikely that all two probes bind on the same wrong site. Concerning the fresh samples of the plants, one could say that the abundance of lactic acid bacteria was apparently not sufficient to be detected, only since lactic acid bacteria propagated throughout the process of fermentation. When looking at the results of the amplicon sequencing the order of *Lactobacillales* however reached abundance rates of 6.8% in *M. chamomilla* L. and 4.6% in *C. officinalis* L. in the course of fermentation. An explanation for the unsuccessful detection of LAB led to the reservation that something in the hybridization process with the probes went wrong. However,

also several attempts with slight variations of the hybridization conditions did not provide a particular result. In the case of the fermented plant samples, maybe the acidic pH influenced the hybridization conditions and did interfere with the binding of the probes.

Genomic fingerprint analysis of antagonistic strains from different habitats and plants

Rep-PCR, among which the BOX-PCR can be assigned to, uses DNA primers which are complementary to highly conserved, repetitive DNA regions naturally occurring in most bacteria. In the case of the BOX-element, a 154 bp sequence is targeted and the amplified fragments are subsequently undergone an electrophoresis analysis. The creation of discriminatory fragment patterns makes it possible to differentiate the initial DNA to species, subspecies and strain level. Due to the used primers cover almost all Gram-positive as well as Gram-negative bacteria, the method is applicable without knowledge of structure or origin of the initial DNA. Evaluation of the band patterns (curve-based or band-based) is done via clustering methods with underlying proximity or reassemble matrixes generating dendrograms as output. Among the band-based analyses, the similarity coefficient by Jaccard evaluates the presence of a band and its position in the gel as a binary variable. Related to this method, the analysis by Dice uses also the band position but the focus thereby lies more on matching bands. The curve-based Pearson's product-moment correlation is based on the densiometric values formed by the fingerprints. An advantage of the curve-based method is the independency of the relative concentrations of the fingerprints and that differences in the background do not affect the results (Rademaker & de Bruijn, 1997).

Some samples did not show any bands as a result of insufficient amplification. Notwithstanding, some of the strains could be grouped together based on their similar electrophoresis band patterns. Especially the samples gathered from the endorhiza of the medicinal plants were grouped together in the created dendrogram.

Deciphering the genome sequence of plant growth promoting bacteria isolated from agriculturally used farmland and arid desert soil in Egypt

Desert farming as it is done in Sekem has converted sparse desert into fertile land. Thereby, not only profitable plants grew well in the transformed new habitat, but with them also soil-borne pathogens emerged, a problem, which was already reported in related circumstances. Among those "uninvited intruders" different taxonomic groups of soil-borne pathogens ranging from fungi (*Fusarium culmorum, Rhizoctonia solani, Verticillium dahliae*), bacteria (*Ralstonia solanacearum*) and also nematodes (*Meloidogyne incognita*) are chronicled (Köberl *et al*., 2013a). The project "BioGuard" in which frame this part of the thesis was realized was launched for "protecting national economic crops via application of beneficial microorganisms to bio-control plant and human pathogens in organic agriculture" especially for farms in Egypt. A plausible but rather ambitious goal of the project purses the development of a biocontrol product to strengthen the plants and promote growth, thereby combating diseases and reducing yield losses. Through the use of beneficial microorganism instead of chemical treatment methods, the sustainability of agriculture can be enhanced and the health of the consumer due to high quality food products (with reduced pathogens and chemical residues) guaranteed.

Problems associated with the sequencing procedure occurred in the DNA isolation of *Streptomyces* sp. Wb2n-11, isolated from the desert soil of Sinai. As a Gram-positive bacterium which are known for their insufficient cell disruption with the common methods (Nikodinovic *et al*., 2003) and maybe also due to its origin – coping with the harsh conditions in the desert soil – enzymatic cell disruption steps based on lysozyme- and mechanical treatment with the ribolyzer weren't sufficient to elicit the strain's DNA in adequate amounts. Several abortive repeats were carried out until the development of the bacterial cell wall was disrupted by the addition of glycine to the growth media, interfering with the peptidoglycan crosslinking of the bacteria. *Streptomyces* sp. Wb2n-11 showed high antagonistic activity in previous analysis against *Verticillium dahlia* and *Rhizoctonia solani* and was also active against *Ralstonia solanacearum* (Köberl *et al*., 2013a) making it a potential strain for further investigations and antibiotic research, as *Streptomyces* is believed the largest antibiotic-producing genus (Watve *et al*., 2001). The sequencing of *Streptomyces* sp. Wb2n-11 and the other two elected strains *Bacillus amyloliquefaciens* Co1-6 and *Paenibacillus polymyxa* Mc5Re-14 was a further step in the characterization of those promising strains. For *Bacillus*

amyloliquefaciens Co1-6 and *Paenibacillus polymyxa* Mc5Re-14, Schmidt *et al.* (2014) showed their ability to alter the metabolism of chamomile plants by enhancing the concentrations of the flavonoids apigenin-7-*O*-glucoside and apigenin. *Streptomyces* sp. Wb2n-11 was among the strains with the highest activity against *Verticillium dahliae* and *Rhizoctonia solani* and showed also antagonistic potential against *Ralstonia solanacearum* (Köberl *et al.*, 2013a).

The sequencing method of PacBio RS II was used taking advantage of the long reads of this technique compared to the second-generation sequencing procedures. Using this method, a polymerase is immobilized onto the end of a zero-mode waveguide (ZMW) called SMRTcell. This assembly enables the observation of amplification in real time through the incorporation of four differently fluorescent-labeled nucleotides, which generate distinct emission spectrums. A double-stranded DNA equipped with hairpin adaptors at both ends thereby forming a closed, single-stranded circular DNA serves as template. The DNA that has been prepared in this way is called a SMRTbell and can be read by the polymerase throughout the replication process. Dependent on the length of the template and the lifetime of the polymerase used, one template can be read multiple times enhancing the accuracy, one of the drawbacks of the PacBio sequencing method. Near the high error rate (especially for long templates), the elevated cost per base and the low throughput are disadvantages of this method. Despite those hindrances, PacBio sequencing can score points with its faster runs and the long-read lengths (up to 60 kb) suitable for *de novo* genome assemblies compared to the second generation sequencing methods (Rhoads *et al.*, 2015).

Abbreviations

°C	degree centigrade
ARDRA	amplified ribosomal DNA restriction analysis
BLAST	basic local alignment search tool
bp	base pair
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
ddH2O	double distilled water
dH2O	distilled water
DMSO	dimethlsulfoxid
DNA	deoxyribonucleic acid
et al.	*et alteri*
FISH	fluorescence *in situ* hybridisation
kb	kilobase
LAB	lactic acid bacteria
min	minute
ON	over night
ONC	overnight culture
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGPBs	plant growth promoting bacteria
rpm	rounds per minute
SSCP	single strand conformational polymorphism
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TEMED	N, N, N, N-tetramethylethylendiamin
UPGMA	unweighted pair group method with arithmetic mean

Appendix

Growth media and solutions

Nutrient medium II (Sifin; Berlin, Germany)

NBII: 15 g
dH_2O: 1000 ml

After water was added to the weighted sample the medium was autoclaved before use.

CASO bouillon (Soy Broth, TSB) (Roth; Karlsruhe, Germany)

CASO bouillon: 30 g
dH_2O: 1000 ml

After water was added to the weighted sample the medium was autoclaved before use.

5 x TBE buffer

Tris [99.9%], (Roth; Karlsruhe, Germany): 54.0 g

Boric acid [99.8%], (Roth; Karlsruhe, Germany): 27.5 g

EDTA [0.5 M], pH: 8: 20 ml

dH_2O was added until reaching 1000 ml of final volume.

Waksman agar

Trypton from Casein (Roth; Karlsruhe, Germany): 5.0 g

Glucose (Roth; Karlsruhe, Germany): 10.0 g

NaCl (Roth; Karlsruhe, Germany): 5.0 g

Yeast extract (Roth; Karlsruhe, Germany): 3.0 g

Agar-agar (Roth; Karlsruhe, Germany): 20.0 g

dH_2O was added until reaching 1000 ml of final volume, pH was set to 6.8 and medium was autoclaved before use.

50 x TAE buffer (Tris-Acetate-EDTA)

Tris [99.9%], (Roth; Karlsruhe, Germany): 242 g

Glacial acetic acid: 57 ml

0.5 M EDTA [0.5 M], pH 8: 100 ml

dH_2O was added until reaching 1000 ml of final volume.

Crush and Soak buffer (50 ml)

EDTA (pH 8.0): 18.50 mg

magnesium acetate: 105.75 mg

ammonium acetate: 1.93 g

SDS (20%): 250 µl

dH_2O was added until reaching 50 ml of final volume.

(according to Sambrook *et al*., 1989)

Fixation protocol and solutions[1] for fluorescent *in situ* hybridization (FISH)

Ethanol fixation

1. Wash sample with PBS
2. Add 1 vol. PBS + 1 vol. EtOH (96%)
3. Store at -20°C
4. Prior to sample observation, wash 3 times with ddH2O

(move from one Eppendorf tube with ddH2O to another with int. 3 min.)

Paraformaldehyde fixation

1. Wash with PBS
2. Add 1 vol. PBS + 3 vol. icecold PFA (4%)
3. Fix at -4°C for 4-12 hours
4. To wash discard PFA from Eppendorf tubes
5. Wash 3 times with cold PBS (1* - rinse, 2* - 5 min., 3* - 10 min.)
6. Add 1 vol. PBS + 1 vol. EtOH (96%)
7. Store at -20°C

[1] Source for the solutions for fluorescence *in situ* hybridisation: International FISH Course 2010, Department of Microbial Ecology, Faculty of Life Sciences, University of Vienna.

4% Paraformaldehyde (PFA) solution (50 ml)

Heat 33 ml distilled water to 65°C, add 2 g PFA while stirring

Add NaOH until paraformaldehyde is dissolved

Add 16.6 ml 3 x PBS

Leave solution cool to room temperature and adjust pH to 7.2 – 7.4

Filter the solution using syringes and 0.2 µm filters

Store solution at -20°C.

Caution: PFA is toxic! Wear gloves and a dusk mask!

Phosphate buffer for PBS

20:80 (v/v) mixture of 200 mM NaH_2PO_4 and 200 mM Na_2HPO_4

pH of the buffer mixture should be 7.2 – 7.4.

3 x phosphate buffer saline (PBS)

390 mM NaCl, 15% (v/v) phosphate buffer

pH of the final solution should be 7.2 – 7.4.

1 x PBS

130 mM NaCl, 5% (v/v) phosphate buffer

pH of the final solution should be 7.2 – 7.4.

Table of all isolated potential LAB

Table 15: List of all 394 isolated potential LAB. Names and numbers indicate the plant source and the varying cultivation conditions.

1	KKS1-1	34	KÖ1-21	74	KKS2-14	96	KÖ2-12
2	KKS1-3	35	KÖ1-22	51	McF1-16	97	McS2-1
3	KKS1-6	36	KÖ1-23	52	McF1-17	98	McS2-2
4	KKS1-8	37	KÖ1-24	53	McF1-18	99	McS2-3
5	KKS1-9	38	McF1-2	54	McF1-19	100	McS2-4
6	KKS1-13	39	McF1-3	55	McF1-20	101	McS2-5
7	KKS1-17	40	McF1-4	56	McF1-21	102	McS2-6
8	KKS1-18	41	McF1-5	57	McF1-22	103	McS2-7
9	KKS1-19	42	McF1-6	58	McF1-23	104	McS2-8
10	KKS1-21	43	McF1-7	59	McF1-24	105	McS2-9
11	KKS1-22	44	McF1-8	60	CoF1-3	106	McS2-10
12	KKS1-23	45	McF1-10	61	KKS2-1	107	McS2-11
13	KKS1-24	46	McF1-11	62	KKS2-2	108	McS2-12
14	KÖ1-1	47	McF1-12	63	KKS2-3	109	McS2-13
15	KÖ1-2	48	McF1-13	64	KKS2-4	110	McS2-14
16	KÖ1-3	49	McF1-14	65	KKS2-5	111	McS2-15
17	KÖ1-4	50	McF1-15	66	KKS2-6	112	McS2-16
18	KÖ1-5	48	McF1-13	67	KKS2-7	113	McS2-17
19	KÖ1-6	49	McF1-14	68	KKS2-8	114	McS2-18
20	KÖ1-7	50	McF1-15	69	KKS2-9	115	McS2-19
21	KÖ1-8	48	McF1-13	70	KKS2-10	116	McS2-20
22	KÖ1-9	49	McF1-14	71	KKS2-11	117	McS2-21
23	KÖ1-10	50	McF1-15	72	KKS2-12	118	McS2-22
24	KÖ1-11	51	McF1-16	73	KKS2-13	119	McS2-23
25	KÖ1-12	52	McF1-17	74	KKS2-14	120	McS2-24
26	KÖ1-13	53	McF1-18	75	KKS2-15	121	McF2-1
27	KÖ1-14	54	McF1-19	76	KKS2-16	122	McF2-2
28	KÖ1-15	55	McF1-20	77	KKS2-17	123	McF2-3
29	KÖ1-16	56	McF1-21	78	KKS2-18	124	McF2-4
30	KÖ1-17	57	McF1-22	79	KKS2-19	125	McF2-5
31	KÖ1-18	58	McF1-23	80	KKS2-20	126	McF2-6
32	KÖ1-19	59	McF1-24	81	KKS2-21	127	McF2-7
33	KÖ1-20	60	CoF1-3	82	KKS2-22	128	McF2-8
34	KÖ1-21	61	KKS2-1	83	KKS2-23	129	McF2-9
35	KÖ1-22	62	KKS2-2	84	KKS2-24	130	McF2-10
36	KÖ1-23	63	KKS2-3	85	KÖ2-1	131	McF2-11
37	KÖ1-24	64	KKS2-4	86	KÖ2-2	132	McF2-12
38	McF1-2	65	KKS2-5	87	KÖ2-3	133	McF2-13
39	McF1-3	66	KKS2-6	88	KÖ2-4	134	McF2-14
40	McF1-4	67	KKS2-7	89	KÖ2-5	135	McF2-15
41	McF1-5	68	KKS2-8	90	KÖ2-6	136	McF2-16
42	McF1-6	69	KKS2-9	91	KÖ2-7	137	McF2-17
30	KÖ1-17	70	KKS2-10	92	KÖ2-8	138	McF2-18
31	KÖ1-18	71	KKS2-11	93	KÖ2-9	139	McF2-20
32	KÖ1-19	72	KKS2-12	94	KÖ2-10	140	McF2-21
33	KÖ1-20	73	KKS2-13	95	KÖ2-11	141	McF2-22

142	McF2-23	177	KGS3-4	200	KÖ3-6	254	KGS4-19
143	McF2-24	178	KGS3-5	201	McS3-1	255	KGS4-20
144	CoS2-1	179	KGS3-6	202	McS3-2	256	KGS4-21
145	CoS2-2	180	KGS3-7	203	McS3-3	257	KGS4-22
146	CoS2-3	181	KGS3-8	204	McS3-4	258	KGS4-23
147	CoS2-4	151	CoS2-8	205	McS3-5	259	KGS4-24
148	CoS2-5	152	CoS2-9	206	McS3-6	260	KKS4-1
149	CoS2-6	153	CoS2-10	207	McS3-7	261	KKS4-2
150	CoS2-7	154	CoS2-11	208	McS3-8	262	KKS4-3
132	McF2-12	155	CoS2-12	209	McS3-9	263	KKS4-5
133	McF2-13	156	CoS2-13	210	McS3-10	264	KKS4-6
134	McF2-14	157	CoS2-14	211	McS3-11	265	KKS4-7
135	McF2-15	158	CoS2-15	212	McS3-12	266	KKS4-9
136	McF2-16	159	CoS2-16	213	CoS3-1	267	KKS4-13
137	McF2-17	160	CoS2-17	214	CoS3-2	268	KKS4-14
138	McF2-18	161	CoS2-18	215	CoS3-3	269	KKS4-15
139	McF2-20	162	CoF2-1	216	CoS3-4	270	KKS4-16
140	McF2-21	163	CoF2-2	217	CoS3-5	271	KKS4-17
141	McF2-22	164	CoF2-3	218	CoS3-6	272	KKS4-18
142	McF2-23	165	CoF2-4	219	CoS3-7	273	KKS4-19
143	McF2-24	166	CoF2-5	220	CoS3-8	274	KKS4-20
144	CoS2-1	167	CoF2-6	221	CoS3-9	275	KKS4-21
145	CoS2-2	168	CoF2-7	222	CoS3-10	276	KKS4-22
146	CoS2-3	169	CoF2-8	223	CoS3-11	277	KKS4-23
147	CoS2-4	170	CoF2-9	224	CoS3-12	278	KKS4-24
148	CoS2-5	171	CoF2-10	225	CoF3-1	279	KÖ4-2
149	CoS2-6	172	CoF2-11	226	CoF3-2	280	KÖ4-12
150	CoS2-7	173	CoF2-12	227	CoF3-3	281	McS4-1
151	CoS2-8	174	KGS3-1	228	CoF3-4	282	McS4-2
152	CoS2-9	175	KGS3-2	229	CoF3-5	283	McS4-3
153	CoS2-10	176	KGS3-3	230	CoF3-6	284	McS4-4
154	CoS2-11	177	KGS3-4	231	CoF3-7	285	McS4-5
155	CoS2-12	178	KGS3-5	232	CoF3-8	286	McS4-6
156	CoS2-13	179	KGS3-6	233	CoF3-9	287	McS4-7
157	CoS2-14	180	KGS3-7	234	CoF3-10	288	McS4-8
158	CoS2-15	181	KGS3-8	235	CoF3-11	289	McS4-9
159	CoS2-16	182	KGS3-9	236	CoF3-12	290	McS4-10
160	CoS2-17	183	KGS3-10	237	KGS4-1	291	McS4-11
161	CoS2-18	184	KGS3-11	238	KGS4-3	292	McS4-12
162	CoF2-1	185	KGS3-12	239	KGS4-4	293	McS4-13
163	CoF2-2	186	KKS3-1	240	KGS4-5	294	McS4-14
164	CoF2-3	187	KKS3-2	241	KGS4-6	295	McS4-15
165	CoF2-4	188	KKS3-3	242	KGS4-7	296	McS4-16
166	CoF2-5	189	KKS3-4	243	KGS4-8	297	McS4-17
167	CoF2-6	190	KKS3-5	244	KGS4-9	298	McS4-18
168	CoF2-7	191	KKS3-6	245	KGS4-10	299	McS4-19
169	CoF2-8	192	KKS3-7	246	KGS4-11	300	McS4-20
170	CoF2-9	193	KKS3-8	247	KGS4-12	301	McS4-21
171	CoF2-10	194	KKS3-9	248	KGS4-13	302	McS4-22
172	CoF2-11	195	KKS3-10	249	KGS4-14	303	McS4-23
173	CoF2-12	196	KKS3-11	250	KGS4-15	304	McS4-24
174	KGS3-1	197	KKS3-12	251	KGS4-16	305	McF4-1
175	KGS3-2	198	KÖ3-4	252	KGS4-17	306	McF4-2
176	KGS3-3	199	KÖ3-5	253	KGS4-18	307	McF4-3

308	McF4-4	330	CoS4-2	352	CoS4-24	374	CoF4-22
309	McF4-5	331	CoS4-3	353	CoF4-1	375	CoF4-23
310	McF4-6	332	CoS4-4	354	CoF4-2	376	CoF4-24
311	McF4-7	333	CoS4-5	355	CoF4-3	377	CoF4-25
312	McF4-8	334	CoS4-6	356	CoF4-4	378	CoF4-26
313	McF4-9	335	CoS4-7	357	CoF4-5	379	CoF4-27
314	McF4-10	336	CoS4-8	358	CoF4-6	380	CoF4-28
315	McF4-11	337	CoS4-9	359	CoF4-7	381	CoF4-29
316	McF4-12	338	CoS4-10	360	CoF4-8	382	CoF4-30
317	McF4-13	339	CoS4-11	361	CoF4-9	383	McF3-1
318	McF4-14	340	CoS4-12	362	CoF4-10	384	McF3-2
319	McF4-15	341	CoS4-13	363	CoF4-11	385	McF3-3
320	McF4-16	342	CoS4-14	364	CoF4-12	386	McF3-4
321	McF4-17	343	CoS4-15	365	CoF4-13	387	McF3-5
322	McF4-18	344	CoS4-16	366	CoF4-14	388	McF3-6
323	McF4-19	345	CoS4-17	367	CoF4-15	389	McF3-7
324	McF4-20	346	CoS4-18	368	CoF4-16	390	McF3-8
325	McF4-21	347	CoS4-19	369	CoF4-17	391	McF3-9
326	McF4-22	348	CoS4-20	370	CoF4-18	392	McF3-10
327	McF4-23	349	CoS4-21	371	CoF4-19	393	McF3-11
328	McF4-24	350	CoS4-22	372	CoF4-20	394	McF3-12
329	CoS4-1	351	CoS4-23	373	CoF4-21		

Abbreviations:
(1) Plant species and location (KKS..."small" cabbage Sekem, KÖ…Austrian cabbage, KGS…"big" cabbage Sekem, CoF…*Calendula officinalis* L. Faiyum oasis, CoS…*Calendula officinalis* L. Sekem, McF…*Matricaria chamomilla* L. Faiyum oasis, McS…*Matricaria chamomilla* L. Sekem
(2) cultivation condition 1–4: 1…R2A aerob, 2…MRS aerob, 3…MRS anaerob, 4…MRS microaerophil
(3) consecutive number of the isolates.

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List of tables

List of figures