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Interaction between silver fir (*Abies alba*) wood water extract and lactobacilli

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Water extract from silver fir (*Abies alba*) wood represents a rich source of lignans and other phenols that are effective in different pathological conditions, such as diabetes, cardiovascular diseases and psoriasis. Its interaction with the gastrointestinal environment is crucial when the extract is orally administered. In this study we tested the *in-vitro* interaction between water extract of silver fir wood and ten different *Lactobacillus* species that are found in the gastrointestinal tract, vagina or are used in food industry. We tested both ways of interaction: 1) the bacterial influence on the chemical composition of the extract and 2) influence of the extract on the bacterial growth. We demonstrated that the extract is compatible with all of the bacteria and does not impair their growth. Furthermore the extract acted as a prebiotic for some bacteria including: *L. paracasei*, *L. acidophilus*, *L. rhamnosus*, *L. gasseri*, *L. crispatus* and *L. bulgaricus*, suggesting that the compounds in the extract can stimulate their growth. However, the ten lactobacilli did not show any chemical changes in lignan metabolism and the production of enterodiol and enterolactone, which are considered the final metabolic products of lignans and are produced by different gut bacteria. This study indicates that the silver fir wood extract is nutritious for some *Lactobacillus* bacteria and can be used as a prebiotic.

1. Introduction

Silver fir (*Abies alba*) is an evergreen tree distributed in the lowlands and the mountains of central as well as in some parts of Southern and Eastern Europe. It is characterized with dark green needle leafs and dark brown bark. The tree can reach heights above 60 meters and can live up to 500-600 years (Mauri 2016). The water extract from the branches of silver fir (Belinal®) contains a variety of chemical compounds of which lignans and other polyphenols are the most abundant (Benkovic et al. 2017). The extract has anti-diabetic activities by inhibiting enzymes involved in regulation of glucose level, e.g α-glucosidase, α-amylase and dipeptidyl peptidase 4 (Lunder et al. 2019). Its anti-diabetic properties were also shown in a double-blind study of 31 healthy individuals, where Belinal® lowered the glucose concentration in the blood after a meal by 35% (Debeljak et al. 2016). Different types of silver fir extracts were also shown to be effective against psoriasis (Zorko et al. 2018) and demonstrated cardioprotective (Drevensek et al. 2016), chondrogenic (Sirse et al. 2020) as well as atheroprotective effects (Drevensek et al. 2015). In addition, the essential oil of silver fir is also known for its beneficial properties (Yang et al. 2009; Lanzerstorfer et al. 2019).

When silver fir extracts are consumed orally, their stability and interaction with the gastrointestinal environment is crucial for their effects. The wood extract of silver fir showed high stability under simulated gastric and intestinal environments (without microbiota), where its lignan content did not decrease after digestion (Benkovic et al. 2017). A lot of studies also explained the correlation between specific phenols and the gut microbiota (Selma et al. 2009; Quartieri et al. 2016; Espin et al. 2017). Many phenolic substances including isoflavones, ellagitannins and lignans act as phytoestrogens showing physiological similarity to the primary female sex hormone, 17-β-estradiol. Their estrogenic effects on the host can be improved through their metabolism by specific gut microbial species which are transforming the phytoestrogens into more lipophilic substances thus enhancing their absorption and affinity to the estrogen receptors (Stojanov and Kreft 2020). The positive interaction can also be observed *vice versa* where specific phenols act as prebiotics and stimulate the growth of specific bacteria (Pacheco-Ordaz et al. 2018; Alves-Santos et al. 2020; Nazzaro et al. 2020; Moorthy et al. 2021). Apart from prebiotic properties, some phenols can promote gut colonization of bacteria by increasing the bacterial adhesion in the intestines (de Souza et al. 2019).

In this study we examined the interaction between water extract of silver fir branches (Belinal®) and ten different *Lactobacillus* species (*L. acidophilus* ATCC 4356, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, *L. casei* ATCC 393, *L. crispatus* ATCC 33820, *L. gasseri* ATCC 33323, *L. paracasei* ATCC 25302, *L. plantarum* ATCC 8014, *L. reuteri* ATCC 55730, *L. rhamnosus* ATCC 53103 and *L. salivarius* ATCC 11741). We tested the lignan metabolic capability of the bacteria as well as the prebiotic properties of silver fir extract. It was previously found that the extract contains six different lignans (isolariciresinol, hydroxymatairesinol, secoisolariciresinol, lariciresinol, pinoresinol and matairesinol) (Benkovic et al. 2017), and that different gut bacteria are able to transform isolated lignans into enterolignans, e.g enterolactone and enterodiol (Heinonen et al. 2001). Some, but not all tested *Lactobacillus* strains (*L. salivarius* INIA P183, *L. salivarius* INIA P448 and *L. gasseri* INIA P508) were found to produce enterodiol and enterolactone also from lignans in flax extracts (Bravo et al. 2017). In our research, we tested metabolic activity of ATCC (American Type Culture Collection) lactobacilli on the lignans from silver fir wood extract, and a prebiotic activity of this extract.

2. Investigations and results

2.1. Lignan metabolism by human microbiota

The ten *Lactobacillus* species did not produce any significant difference in the silver fir extract or in the MRS media (Fig. 1). The bacteria did not yield any changes and production of metabo-

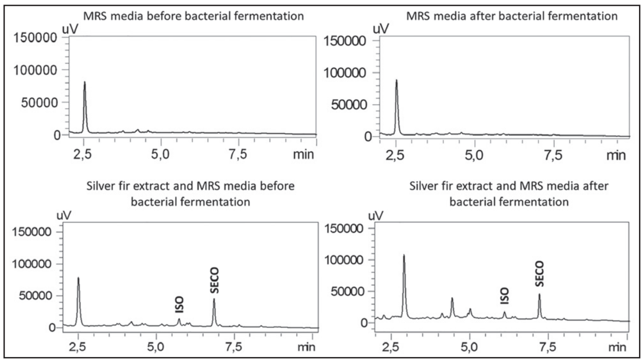

Fig. 1: HPLC graphs of MRS media alone or in combination with silver fir extract, before and after bacterial fermentation. MRS: De Man, Rogosa and Sharpe; ISO: Isolariciresinol; SECO: Secoisolariciresinol

lites similar to lignans in the MRS media after fermentation. The two lignans (isolariciresinol and secoisolariciresinol) found in the silver fir extract, did not undergo metabolic changes and no production of enterodiol or enterolactone was detected.

2.2. *Growth characteristics*

The ten bacterial strains showed divergent growth when cultured with or without the extract of silver fir. The differences were with regard of maximal optical density (max OD), lag time and growth rate. *L. salivarius* showed the highest max OD of 1.46 while the lowest max OD was achieved by *L. gasseri* 0.24. Similarly, *L. salivarius* showed the fastest growth rate of 0.264/h, while *L. bulgaricus* was the slowest grower with a growth rate of 0.035/h. On the other hand, the lag time was longest in *L. crispatus,* 14.4 h, whereas in *L. paracasei* it was 1.6 h.

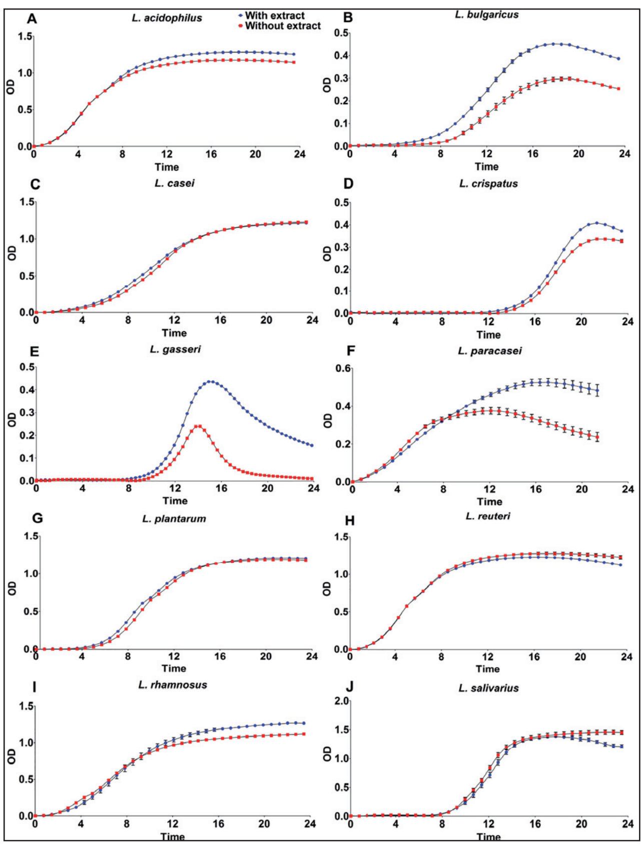

Fig. 2: Growth curves of different *Lactobacillus* species with and without silver fir extract. A: *L. acidophilus*, B: *L. bulgaricus*, C: *L. casei*, D: *L. crispatus*, E: *L. gasseri*, F: *L. paracasei*, G: *L. plantarum*, H: *L. reuteri*, I: *L. rhamnosus,* J: *L. salivarius*. Blue lines: bacteria plus extract, red lines: bacteria only.

2.3. *The effects of silver fir extract on the growth of Lactobacillus species*

The growth curves of all bacteria with and without the extract are shown in Fig. 2. For better visualization only values up to 24 h were taken. From the graphs it is seen that silver fir extract influences differently the growth of different bacteria when cultured under the same conditions. The extract increases the growth of several bacteria including: *L. acidophilus* (Fig. 2A), *L. bulgaricus* (Fig. 2B), *L. crispatus* (Fig. 2D), *L. gasseri* (Fig. 2E), *L. paracasei* (Fig. 2F) and *L. rhamnosus* (Fig. 2I). Non-significant growth inhibition was observed in *L. reuteri* (Fig. 2H) and *L. salivarius* (Fig. 2J). Silver fir extract did not influence the growth of *L. casei* (Fig. 2C) and *L. plantarum* (Fig. 2G).

The analysis of max OD, growth rate and lag time revealed a statistical difference between different species when cultured with silver fir extract. Only in *L. salivarius* we observed a significantly lower max OD when cultured with silver fir extract (p<0.01). Other bacteria such as: *L. paracasei*, *L. acidophilus*, *L. rhamnosus*, *L. gasseri*, *L. bulgaricus* and *L. crispatus* showed higher max OD in the presence of silver fir extract. The difference in max OD in other bacteria was not significantly different (Fig. 3).

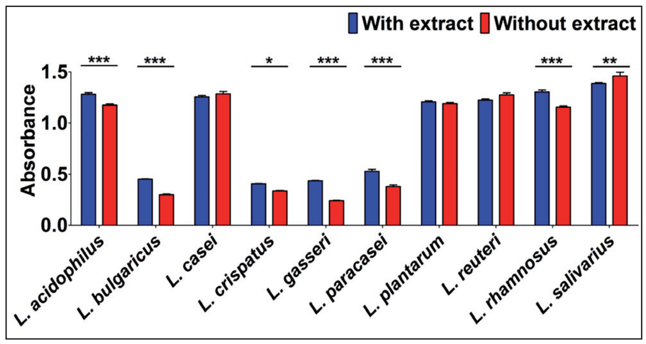

Fig. 3: Maximal optical density of different *Lactobacillus* species with and without silver fir extract. *(p<0.05), **(p<0.01), ***(p<0.001).

The extract influenced the duration of lag time in some bacteria. Reduction in lag time allows the bacteria to reach its exponential and stationary phase faster and thus promote its activity faster. In our work we concluded that the extract of silver fir reduced the mean lag time in *L. gasseri* and *L. bulgaricus* by 3.0 h and 1.6 h, respectively. However, in *L. rhamnosus* the extract increased the duration of lag time by 1.1 h. The silver fir extract did not show any significant difference in lag time duration in other bacteria (Fig. 4).

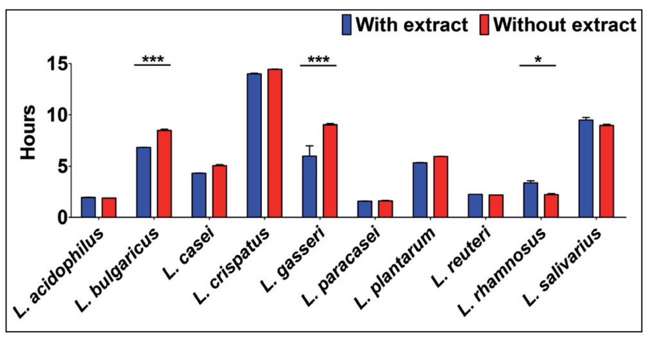

Fig. 4: Lag time of different *Lactobacillus* species with and without silver fir extract. *(p<0.05), **(p<0.01), ***(p<0.001).

Figure 5 shows the growth rate of the ten *Lactobacillus* species, of which only in *L. gasseri* and *L. rhamnosus* the growth rate was significantly increased when cultured together with silver fir extract. The growth rate in *L. gasseri* was increased by 0.037/h and in *L. rhamnosus* by 0.020/h.

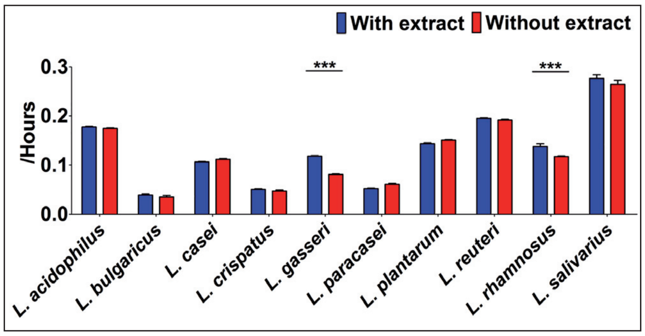

Fig. 5: Growth rate of different *Lactobacillus* species with and without silver fir extract. *(p<0.05), **(p<0.01), ***(p<0.001).

3. Discussion

Silver fir extract contains a variety of different lignans (Benkovic et al. 2017). Their beneficial health properties were demonstrated in several *in vitro* and clinical studies (Debeljak et al. 2016; Lunder et al. 2019). Even though lignans are well known for their antibacterial properties against Gram-positive and Gram-negative bacteria (Favela-Hernandez et al. 2012; Kyselka et al. 2017), their effects on human microbiota have not been thoroughly studied. In our work, we examined the interaction between silver fir wood extract and ten different *Lactobacillus* species, several of them being human isolates. We concluded that the silver fir extract acted as a prebiotic for several gut bacteria including *L. paracasei*, *L. rhamnosus* and *L. acidophilus*, vaginal bacteria *L. gasseri* and *L. crispatus*, and also bacteria used in food industry – *L. bulgaricus*. The exact compound or compounds influencing the bacterial growth are unknown. Apart from phenols, other non-phenolic compounds such as carbohydrates are also found in the silver fir extract (Benkovic et al. 2017). A lot of carbohydrates especially different oligosaccharides can increase the growth of several bacteria and are also considered as prebiotics for different human bacteria (Davani-Davari et al. 2019; Guarino et al. 2020).

Furthermore, different lactobacilli can influence the metabolism of specific lignans (Bravo et al. 2017; Stojanov and Kreft 2020) and produce more active compounds with greater therapeutic effects. However, in our experimental conditions the tested strains did not show any metabolic effects regarding the production of enterodiol and enterolactone from lignans found in silver fir extract. In our experiments the concentration of lignans was lower compared to published experiments, where metabolic activity was observed, and bacterial strains were different. It is important to state that biological effects of probiotics vary between different strains, therefore failure or success of one strain cannot be applied to other strains (Fijan 2014). The metabolism of lignans by certain strains depends on their ability to produce different enzymes such as: b-glucuronidases, sulfatases and glucosidases (Possemiers et al. 2011). Apparently, the strains in our research do not produce the specific enzymes responsible for metabolism of isolariciresinol and secoisolariciresinol and production of enterodiol and enterolactone.

All of the ten species included in this study are well known for their probiotic characteristics. Addition of prebiotics can increase the growth of probiotics and thus promote their therapeutic effects. In this study we concluded that the silver fir extract (Belinal®) is compatible with the bacteria and also, in some cases, it shows prebiotic properties. This study indicates that the use of *Lactobacillus* probiotics together with silver fir wood extract could have positive impact and improve the beneficial effects of probiotics.

4. Experimental

4.1. Bacterial culturing and fermentation

Ten different *Lactobacillus* species were used in this study (*L. acidophilus* ATCC 4356, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, *L. casei* ATCC 393, *L. crispatus* ATCC 33820, *L. gasseri* ATCC 33323, *L. paracasei* ATCC 25302, *L. plantarum* ATCC 8014, *L. reuteri* ATCC 55730, *L. rhamnosus* ATCC 53103 and *L. salivarius* ATCC 11741). All of the bacteria were kept frozen at -80 °C in De Man, Rogosa and Sharpe (MRS) obtained from Merck (Darmstadt, Germany) with 20% glycerol for long-term storage. Fresh bacteria cell cultures were transferred to MRS media solidified with 1.5% agar and were grown at 37 °C for 48 h in anaerobic gas generating bags (GasPak™ EZ) obtained from Becton, Dickinson and Company. Single colony was inoculated into 5 ml MRS liquid medium along with 20 mg/l of silver fir extract. For each bacterium, we prepared six different inoculants of which three were incubated at 37 °C for 24 h and three for 48 h. Afterwards, the cultures were centrifuged at 4400x g for 10 minutes (Eppendorf centrifuge 5702 r) and the supernatant was decanted in fresh falcon tubes and stored at -20 °C.

4.2. Extract preparation

Belinal® extract was obtained from Abies Labs, Ljubljana, Slovenia. It is a dried aqueous extract from the silver fir (*Abies alba*) wood from branches (Benkovic et al. 2017).

4.3. HPLC analysis

The HPLC system (Shimadzu Prominence) consisted of a system controller (CBM-20A), a column oven (CPO-20AC) and a solvent delivery pump with a degasser (DGU-20A5) connected to a refrigerated autosampler (SIL-20AC) with a photodiode array (PDA) detector (SPD-M20A) that monitored the wavelengths 190–800 nm. The responses of the detector were recorded using LC Solution software version 1.24 SP1. The chromatographic separation was performed at 40 °C and a flow rate of 2 mL/min using a Phenomenex Kinetex® XB-C18 column (10 cm × 4.6 mm I.D., 2.7 µm particle size). The gradient elution method using solvent A (2% acetonitrile and 0.1% trifluoroacetic acid in water) and solvent B (2% water and 0.1% trifluoroacetic acid in acetonitrile) was used: 0–1 min 5% B, 1–10 min 5–30% B, and 10–15 min 100% B (Benkovic et al. 2014).

4.4. Bacterial survival in the presence of silver fir extract

Fresh over-night cultures of *Lactobacillus* bacteria were diluted (1:100) in two 5 mL aliquots of MRS media. The silver fir extract was added (to 1 mg/l final concentration) in the first aliquot, while the other served as a control with only bacteria in MRS. Each stain was grown in pentaplicate of 200 ml in 96-well microplates which were sealed with sealing film and incubated in a microplate reader (Sunrise; Tecan, Salzburg, Austria) at 37 °C for 50 h as previously described (Zupancic et al. 2019). The parameters of the growth curves were analyzed using DMFit 3.5 software and the model of Baranyi and Roberts (1994).

4.5. Statistical analysis

The bacterial survival and the effects of silver fir extract were statistically analyzed with GraphPad Prism 5.00 software using analysis of variance (ANOVA) and Bonferroni post hoc test. The results are presented as means±standard deviation (SD).

Conflicts of interest: None reported.

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