B. Scientific report

Slutrapport för projekt finansierat av Ekhagastiftelsen
Diarienummer: 2016-85
Projekttitel: Näringsrika och smakliga fermenterade svenska ekologiska baljväxter
Anslagsmottagare: Chalmers tekniska högskola, Livsmedelsvetenskap
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Introduction

Fava bean is a protein crop, suitable for Swedish climate, with favorable nutritional composition and good pre-fruit value, and has together with other legumes been highlighted as healthy and sustainable alternatives to animal protein. Fermentation of fava beans provide better bioavailability of nutrients as well as it can improve palatability and the sensory properties in the product. Fermentation can lower the need for additives in the final product, which is highly favorably in an organic context where the use of additives is low. Within the non-dairy segment, there is a need to develop new starter cultures which are aimed for a non-dairy base such as fava bean beverage. In order to introduce new microorganisms on the food market, it is important to characterize the strains and make sure that these are non-pathogenic and suitable for consumption.

The aim of this study was to identify and characterize and evaluate a number of lactic acid bacteria that, during previous work, were isolated from legumes cultivated on Gotland, and the ability of these bacteria to ferment a fava bean beverage without an additional carbon source. By creating added value to Swedish organic legumes and contributing concrete data to the food industry, we want to promote Swedish organic farming.

Our hypotheses were:

- The isolated bacteria are different strains of lactic acid producing bacteria
- The strains, isolated from legumes, will be able to metabolize the complex carbohydrates in legumes and thus be able to ferment legumes with no additional added carbon source or additives
- The strains will hold properties that indicate that they have probiotic potential
- The strains are not resistant towards the most clinically relevant antibiotic substances

Methods and material

Plant samples

A total of 23 samples were collected from fields and from stored seeds. The 12 field samples were picked from five different locations on Öland, Sweden. On each site, approximately 50g was collected by picking whole pods directly from the plant with sterile gloves. The samples were placed into clean bags and stored at 4°C until analysis. The reminding 11 samples were seeds that had been harvested on Öland, dried and stored in 4°C for 8 months by the producer. The samples were different varieties of *Phaseolus vulgaris*; black beans, brown beans, kidney beans, navy beans, borlotti beans and yin yang, and two varieties of peas; *Pisum sativum*. All samples came from plants cultivated 2015 on Öland.

Isolation of lactic acid producing bacteria

For the selection of LAPB from the Öland beans, roughly one gram of plant sample was inoculated in MRS broth (Sigma-Aldrich, Missouri, USA) with an adjusted pH of 5.7 using HCl (Sigma-Aldrich). The flasks were incubated at 39°C for 4 days in a shaking incubator (Barnstead Lab-Line, ON, Canada) at 190 rpm. Dilutions of the liquid were spread in parallels on MRS agar (Scharlab, Spain) plates, these were then incubated anaerobic using Anaerocult A (Merck Millipore, Germany) at 39°C for 72h. Surviving colonies were picked out and re-streaked on MRS agar plates until isolated bacterial strains were obtained. Isolation was made two subsequent times per sample.

Identification of isolates as lactic acid bacteria

Colonies from all isolates were tested biochemically. Only isolates that were gram positive, oxidase negative and catalase negative and were selected for further analysis. These isolates were harvested for DNA extraction and stored in -80°C.

Rep-PCR *genomic fingerprinting*

Genomic DNA was extracted from the isolates by the protocol previously described by F. Sjöberg et. al {SJöberg, 2013 #55}. One loop of bacteria incubated in 39°C for 74h on horse blood agar plates (Scharlab) were inoculated in 50µl lysis buffer (10 mmol l^{-1} Tris HCl, 1 mmol l^{-1} EDTA and 10 mmol l^{-1} saline (Sigma-Aldrich)) and incubated 10 minutes in 95°C following centrifugation for 5 minutes at 12,000 x g. The supernatant was then collected, measured for DNA concentration using NanoDrop spectrophotometer and stored in -20°C until use.

Strain diversity were assessed by repetitive polymerase chain reaction (rep-PCR), using the single nucleotide $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3') (Invitrogen, California, USA) {Versalovic, 1998 #84}. Reactions were performed using 2720 Thermal cycler (Applied Biosystems, California, USA). The amplification program had an initial denaturation of 98°C for 3 minutes, followed by 30 cycles of denaturation (98°C, 30 sec), annealing (40°C, 1 min), extension (65°C, 8 min) and a final single extension (65°C, 16 min). Each 25 µl PCR reaction contained 1x HF Phusion PCR buffer (Thermo Fisher, Massachusetts, USA), 2.5 mmol l^{-1} $MgCl_2$ (Thermo Fisher), 170 µg m L^{-1} BSA (Thermo Fisher), 200 µmol l^{-1} dNTPs (Thermo Fisher), 2 µmol l^{-1} $(GTG)_5$ primer (Invitrogen), 0.05 U Phusion polymerase (Thermo Fisher), 5% DMSO (Thermo Fisher) and 60 ng of template DNA.

Amplification products were resolved by electrophoresis on a 25 cm gel in a 40 cm cell (Bio-Rad, Sub Cell GT, California, USA) at a constant voltage of 1.5V/cm for 16 hours, in 4mm 1.5 % (w/v) agarose (Thermo Fisher) gel in 1 x TBE buffer (Sigma-Aldrich). A DNA

molecular weight ladder (Thermo Fisher) was used as a reference. Gels were stained post run with 0.1 M NaCl in 3X GelRed (Biotium, California, USA) and visualized by UV light using a Gel Doc (BioRad).

The PCR fingerprints were analysed using BioNumerics (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarity of digitalized band patterns was calculated using DICE coefficient. Complete linkage algorithms were used to construct an average linkage dendrogram to show relationship of isolates.

MALDI-TOF MS identification

The 116 isolates were analysed twice with MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry) analysis using intact cell biomass obtained from overnight cultures of lactic acid producing bacteria at 37 °C on horse blood agar plates. Samples were spotted in duplicates on disposable target plates (bioMeriéux, Marcy l'Etoile, France) and overlaid with 1µl VITEK MS-FA (Formic acid, bioMeriéux), air-dried and again overlaid with 1µl VITEK MS-CHCA matrix solution (α-cyano-4-hydroxycinnamic acid, bioMeriéux). MALDI-TOF MS analysis was performed using a VITEK MS instrument (bioMeriéux), in the range of 2000-20000 m/z, and the spectral data analysed using the VITEK MS IVD and VITEK MS SARAMIS/RUO databases (bioMeriéux).

Phenotypic tests related to probiotic potential

Bile tolerance

Tolerance of the isolates to bile salts was assayed as described by Kumar *et al*. with slight modifications [19]. Unique strains were propagated twice in MRS broth for 24h in 37°C, collected by centrifugation (5000g, 5 min) and washed twice in PBS (Thermo Fisher). The OD_{600} was then adjusted to 0.4-0.6 and then 200µL of the diluted cells were inoculated into duplicates of 8mL of MRS with 0.3% (w/v) bile salts (Sigma Aldrich). Cells were then incubated in a shaking incubator at 37°C. Bacterial enumeration was performed using five replicates per dilution on MRS agar plates at 0h, 24h and 48h.

Phenol tolerance

The isolates ability to survive in the presence of phenol was examined according to the method of Xanthopoulos *et al* {Xanthopoulos, 2000 #200}. Unique strains were prepared in the same way as described for bile tolerance test. The OD_{600} was then adjusted to 0.4-0.6 and then 200µL of the diluted cells were inoculated into duplicates of 8mL of MRS with 0.4% phenol (Sigma Aldrich). The cells were then incubated in a shaking incubator at 37°C. Bacterial enumeration was performed using five replicates per dilution on MRS agar plates at 0h and 24h.

Low pH tolerance

Unique strains were prepared in the same way as described for bile tolerance test. After propagation and dulution, cells were inoculated in triplicates to 8mL of liquid MRS adjusted to pH 2.5. Cells were then incubated at 37°C with continuous stirring using a tube rotator (New Brunswick scientific, New Jersey, USA). Bacterial enumeration was performed using five replicates per dilution on MRS agar plates at 0h, 0,5h, 1h, 1.5h, 2h and 2.5h.

Qualitative determination of bile salt hydrolases activity (BSH)

The taurodeoxycholic acid (TDCA) hydrolase activity was determined for the unique strains using 0.5% (w/v) bile salt-MRS agar plate assay {Dashkevicz, 1989 #197}. MRS agar plates

were supplemented with 0.5% TDCA (Sigma Aldrich).

Bile salt-MRS agar plates were plated in duplicates with each representative strain and incubated anaerobically using an Anaerocult A at 37°C for 72h in before observation. A precipitation zone surrounding colonies indicated BSH activity.

Screening of isolated *Enterococcus* strains for antibiotic resistance

Isolates identified as *Enterococcus* species were tested for antibiotic resistance using disk diffusion test according to standard protocol. The isolated *Enterococcus* were tested for Ampicillin, Trimethoprim, Vancomycin and Nitrofurantoin.

Production of bean flour

Dried fava beans were soaked overnight in deionized water with 2% (w/v) $NaHCO_3$ (Sigma Aldrich), peeled by hand and incubated in 95°C deionized water with 0.5% (w/v) $NaHCO_3$ for four minutes in a water bath. The boiled beans were then blended in 90°C deionized water for 60 seconds, and put on an ice bath to cool before freeze-drying. The freeze-dried flour was then stored in -18°C until use.

Fermentation of fava bean flour medium

A bean flour medium was prepared using deionized water with 6% (w/v) fava bean flour brought to a boil, pH adjusted to 6.2, filtered through a cheese cloth and then autoclaved. Selected strains belonging to *P. pentosaceus* were incubated over night on MRS plates in 37°C and diluted in PBS to an OD_{600} of 0.5-0.8. In triplicates, 8 mL of the fava bean medium was inoculated with 200 µL of diluted cells from five selected strains. Bacterial enumeration

was performed using five replicates per dilution on MRS agar plates at 0h, 4h, 6h, 8h, 12h, 24h and 48h.

Results

Isolation of LAPB

A total of 116 isolates of lactic acid producing bacteria were originally obtained from the 23 samples. Strains of LAPB were isolated from each sample, apart from the black bean samples where no LAPB were isolated.

Rep-PCR and MALDI- TOF MS identification

Rep-PCR fingerprints from all 116 isolates were clustered together into five main groups, fingerprints that showed >90% similarity were considered identical. From each cluster of identical strains, one strain were randomly picked out as a representative for further analysis, resulting in a total of 25 unique strains. MALDI-TOF MS identified the strains as *Enterococcus hirae, Enterococcus faecium, Enterococcus mundtii, Bacillus coagulans* and *Pediococcus pentosaceus.*

Bile tolerance

Strains belonging to *E. faecium* showed a high level of tolerance to 0.3% bile acid, along with strains belonging to *E. mundtii* and *P. pentosaceus*, with some exceptions. *E. hirae* strains

and most *B. coagulans* exhibited a low bile tolerance with a depleted or halved CFU level after 48h of incubation.

Tolerance to low pH

B. coagulans strains showed a high degree of tolerance and had an exponential growth during incubation. Strains *P. pentosaceus* 77, 100, 101 and 176 showed tolerance towards low pH. Strains belonging to *B. coagulans* and *E. faecium* showed a low tolerance level to pH 2.5, and were almost completely depleted after one hour of incubation.

Phenol tolerance

In general, strains from all genuses showed tolerance to 0.4% phenol with a few exceptions.

Qualitative determination of bile salt hydrolases activity (BSH)

Isolated strains from *P. pentosaceus* and *B. coagulans* showed no BSH activity, while all strains from *E. mundtii and E. faecium* were positive for BSH activity. Among *E. hirae,* both BSH negative and BSH positive strains were present.

Antibiotic resistance

From the 11 *Enterococcus* strains that were screened, a total of four strains showed susceptibility towards all tested antibiotics. Of the remaining strains, three showed resistance towards one or more of the tested antibiotic substances and four showed intermediate resistance to at least one of the tested antibiotics.

Fermentation of fava bean flour medium

Isolated *P. pentosaceus* strains were able to grow in 6% fava bean medium without additional carbon added. The strains showed homogenous growth pattern with an initial lag phase following exponential growth. All strains reached a stationary phase after 8h of incubation.

Discussion

There are strong arguments to change the food patterns in industrialized countries into more healthy and sustainable ones. Diets with a low environmental impact are high in plant based food sources, and low in animal protein. Industrial livestock production, especially ruminant meat and dairy products, is pointed out as the main source of GHG emissions from the food sector in industrialized countries, with a high resource footprint. Pulses, such as beans and lentils, are often highlighted as a nutritious and sustainable protein source. In the Nordic region, the vast majority of the pulses consumed are imported from other regions despite the possibility to cultivate locally produced varieties such as fava beans, common beans, sweet lupine, peas and lentils. To promote consumption of Nordic protein crops, there is a need to develop novel products based on these raw materials, for example as alternatives to dairy products. Few probiotic non-dairy products exist on the market world wide, despite the growing demand for dairy-free and animal-free products. Thus it is important to identify strains that are able to ferment non-dairy media, both probiotic and non-probiotic. Swedish pulses could be a source for obtaining strains adapted to the complex carbohydrates in beans and lentils, potentially with probiotic characteristics, that are able to ferment a fava bean beverage.

In this study, 116 lactic acid producing bacteria was isolated, reduced to 25 unique strains from five different genuses by rep-PCR and MALDI-TOF MS. Following identification, the 25 unique strains were characterized in vitro for selected recognized probiotic potential tests evaluating BSH activity, bile- phenol- and low pH-tolerance, and antibiotic resistance.

Another relevant test included in this study was BSH activity. The ability to produce BSH enzyme and thus detoxify bile salts has often been included as a critera for probiotic strain selection. BSH catalyze the deconjugation of bile salts to liberate free bile acids. Strains belonging to *E. mundtii* and *E. faecium* were all able to hydrolyse the bile salt taurodeoxycholic acid, along with strain 78, belonging to *E. hirae*. BSH activity infer that isolates not only survived toxicity of these salts, but also carry out deconjugation of these salts and may help in their intestinal colonization and aid in reducing the blood cholesterol level in the host.

For the bile tolerance test, a concentration of 0.3% bile salts were used, which is similar to the concentration found in the small intestine. In this study, strains belonging to *E. faecium, E. mundtii* and *P. pentosaceus* showed a high level of tolerance towards 0.3% bile. Both bile- and low pH-tolerance tests are important since they indicate the survival, and functional effectivity in the passage, through the stomach - a crucial step for probiotic organisms. Among the strains isolated in this study, strains belonging to *B. coagulans* and *P. pentosaceus* strains 77, 100, 101 and 176 showed tolerance towards pH 2.5, indicating that these strains potentially can survive the harsh environment of the stomach.

Tolerance to phenol was another relevant probiotic characteristic evaluated, since phenols may be formed in the gut by bacterial deamination of aromatic amino acids derived by dietary or endogenously produced proteins. Phenols, and certain other products of protein degradation in the colon such as ammonia, indoles and amines, exert toxic effects in animal models and *in vitro*. These compounds are present in fecal samples and can exert bacteriostatic effect against some lactic acid producing bacteria.

Antibiotic resistance was examined for *Enterococcus* strains because of the high occurrence of resistance within the *Enterococcus* genus. Among the *Enterococcus* strains, four were susceptible to all four of the tested antibiotics; *E. faecium* 63 and *E. mundtii* 94, 146 and 189.

Finally, five selected strains belonging to *P. pentosaceus* were tested for their ability to grow in a fava bean medium with no additional carbon, indicating their potential in fermenting a bean beverage. The five strains had a similar growth pattern, with an initial lag phase followed by exponential growth until 8h of incubation where the cells reached a stationary phase. The cells were able to utilize nutrients from fava beans, making them potential for further investigation and use within the food industry.

In conclusion, four of the strains isolated in this study showed three different *in vitro* properties that make them potential candidates for probiotic applications. Two of these strains, *P. pentosaceus* 77 and 176, have the ability to ferment a fava bean beverage and exhibits excellent bile-, phenol- and low pH tolerance. The other two, *E. faecium* 63 and *E. mundtii* 146 show BSH activity along with bile- and phenol tolerance. These results suggest that strains 77, 146, 77 and 176 have promising properties that are important for potential probiotics. Strains 77 and 146 are further more interesting from a fermentation point of view. More research is needed to exploit other potential probiotic properties of these strains, and further investigate the possibility of the fermentation effects of *P. pentosaceus* on non-dairy substrates.

List of publications

C. Mayer Labba, T. Andlid, Å. Lindgren, A-S. Sandberg, F. Sjöberg, Isolation, identification and characterization of Lactic Acid Producing Bacteria from Swedish grain legumes *Phaseolus vulgaris* and *Pisum sativum*. Submitted Dec. 2018 to Journal of Applied Microbiology