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Comparison of Biochemical Characteristics of Lactobacilli Isolates from Food and Bovine Rumen Sources under Low pH and Bile Salt Stress Conditions

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In this study, two lactobacilli isolates, from dairy food and bovine rumen, were used to investigate the differences in their biochemical characteristics when exposed to low pH and bile salt stress conditions. Production of volatile compounds was analysed by gas liquid chromatography (GLC), alterations in fatty acid profiles of bacterial cells by chromatography-mass spectrophotometry (GC-MS), and changes in protein banding patterns were detected using 1D SDS PAGE. Significant differences were found in the production of acetic acid by stressed and non-stressed cells. Hexanoic acid and octanoic acid production was observed by the cells exposed to bile salt stress only. For both isolates, the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) levels were significantly different for all treatments, except however an increased level of polyunsaturated fatty acids (PUFA) production under bile salts stress was observed. The comparison of the 1-D protein banding patterns of non-stressed and pH stressed and bile salts stressed cells revealed several protein bands were differentially expressed. Findings of this investigation suggest that lactobacilli isolates possess variation in biochemical characteristics on the basis of origin and in their response to low pH and high bile salt stress conditions.

1. Introduction

Lactobacilli have been isolated from fermented foods, gut of animals, faecal content etc. for applications in industrial biotechnology and health (as probiotics). It is a common assumption that lactobacilli from natural/ indigenous origin possess better potential probiotic properties. Lactobacilli are a group of bacteria, which have great diversity in their habitats, genomics, physiological and biochemical characteristics. Moreover, lactobacilli isolates from different origin may possess unique characteristics and display variations in response to environmental stress conditions (Hosseini Nezhad et al., 2015). These responses are also known to be stress specific such as starvation (Hussain et al., 2006).
Lactobacilli produce volatile fatty acids (VFA) as a result of anaerobic fermentation of complex carbohydrates. The nature of the substrate plays a key role in determining the type of VFA compounds produced. Ruminants are known to depend on VFA compounds to meet majority of their maintenance energy requirements. Acetic acid, propionic acid and butyric acid are known for their role in stimulation of sodium and fluid absorption by the colon (Tagang et al., 2010). With regard to the role of VFA compounds from probiotics, the ability to impart flavours to food products is of interest. However, only few reports are available on flavour biochemistry of lactobacilli under stress conditions (Hussain et al., 2009b). Additionally, the fatty acid profiles of bacterial cells are species specific. Depending on the degree of saturation of the carbon chain fatty acids can be divided into three classes: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Saturated fatty acids do not have a double bond. Monounsaturated fatty acids contain only one double bond and PUFA contain two or more double bonds. However, stress conditions and environmental factors can induce a change in the fatty acid profiles. In this case, low pH and increased bile salts concentration have been used to create such stress conditions for lactobacilli. There is limited literature

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reporting whether the stress induced changes are essential for survival and also whether the ability to alter the fatty acids composition is predetermined (Suutari et al., 1990).
Proteomic studies are advantageous to investigate functional molecules and not just the source code, unlike genomics. Proteomic approaches comprises of in silico analysis of predicted protein sequences (van de Guchte et al., 2012). Proteins are produced in abundance by an organism and they are highly diverse in their properties. The protein expression profile of an organism under standard growth conditions and stressful environmental conditions varies widely. It is not possible to study and analyse the entire proteome in one single step. Therefore, entire proteome is divided into different protein subsets comprising; intracellular proteins, membrane proteins or extracellular proteins (Garrigues et al., 2005). In the field of probiotics, proteomic tools have been used primarily for focussing on bacterial stress responses encountered during food production and processing conditions. Variations in temperature, presence of bile salts, acidic environment, oxidative stress, presence of NaCl, nutrient limitation are examples of stress conditions which are commonly used. The stress response of an organism is highly complex and unique to that particular organism. It is also influenced by the environmental conditions and experimental setup. This study compares the biochemical characteristics of two lactobacilli isolates, one each from dairy food and the bovine rumen, by investigating the production of volatile compounds, alterations in fatty acid profiles and changes in the 1D SDS PAGE patterns under low pH and high bile salt stress conditions. This information could be useful to predict the industrial potential of the isolates.

2. Materials and Methods

2.1 Bacterial isolates and growth conditions

Bacterial isolates used in this study were *L. rhamnosus* MI 13 (isolated from a dairy food product) and *L. plantarum* RC 2 (isolated from the bovine rumen) as reported by Jose et al. (2015). Glycerol stock cultures (0.1 mL) of RC 2 and MI 13 were inoculated into MRS broth of pH 6.4 (phosphate buffered). It was mixed well and incubated at 37 °C overnight. The following day, the overnight culture OD was measured at 600 nm. The overnight culture was inoculated at an initial OD of 0.3 into MRS broth (X mL) containing two conditions of stress: pH 3.5 and 3.5 % bile salts. The inoculated broths were incubated at 37 °C for 16 h. Overnight culture was also added to standard MRS broth at an initial OD of 0.15. Cells were harvested once the OD reached 1.5 - 2.0 after approximately 8 - 10 h or until pH was consistently greater than 5. Further incubation was avoided as this can lead to a drop in the pH, which can induce an additional stress.
For VFA and FA profile analysis, after incubation the bacterial cultures were centrifuged at 10,000 rpm for 10 min at room temperature. The cell free supernatant (CFS) was transferred into smaller 1.5 mL eppendorf tubes and stored at -20 °C until gas chromatography-mass spectrophotometry (GC-MS) was carried out for detecting the VFA compounds. Whereas the pellet was washed in 0.1 % in peptone water and again centrifuged at 10,000 rpm for 10 min. This step was repeated three times and the final pellet was transferred into 0.5 mL eppendorf tubes and stored at -20 °C until sample preparation for GLC analysis. For 1D SDS PAGE, after centrifugation cells pellets were finally suspended 40 mM Tris buffer to achieve an OD of 20 ± 0.1 at absorbance of 600 nm, transferred into 1.5 mL screw capped vials and stored at -20 °C until further use.

2.2 Identification of VFA

The analysis of VFA compounds in bacterial broth was determined using an automated Headspace Solid-Phase Micro-Extraction (HS-SPME) based on the method published by Tomasino et al. (2015). Sample preparation involved pipetting 0.45 mL of broth, 0.45 mL of deionised water and 8.06 mL of 5 g/L tartaric acid buffer (pH 3.5) into 20 mL SPME sample vials (a 20 fold dilution of the broth), followed by 40 µL of the internal standard solution. Sodium chloride (4.5 g) was then added to the SPME vial and capped. Samples were incubated initially for 10 min at 50 °C during which time the vial was agitated at 500 rpm. After 10 min the SPME fibre (2 cm long Stableflex DVB/CAR/PDMS, p/n 57348-U, Sigma-Aldrich Australia) was exposed to the headspace of the vial for a period of 30 min at 50 °C. During this exposure period the headspace volatiles were adsorbed onto the fibre. Desorption of these volatiles occurred when the fibre was inserted into the GC injection port for 5 min at 250 °C. Prior to use the SPME fiber was conditioned at 270 °C in the injection port for 1 h. Before each sample was run the SPME fiber was conditioned for 10 min at 270 °C in a fibre conditioning station attached to the Combi-Pal auto sampler used with the Shimadzu GC-MS (model GC-MS-QP2010) with GC-MS solution version 2.72 for data acquisition. Helium was used in the fiber conditioning station to create an oxygen free atmosphere.
The chromatography was performed using two GC columns in series namely a Rtx-Wax 30.0 m × 0.25 mm ID × 0.5 µm film thickness (Polyethylene Glycol-Restek, Bellefonte, PA, USA) and a Rxi-1MS 15 m × 0.25 mm ID × 0.5 µm (100 % dimethyl polysiloxane-Restek, Bellefonte, PA, USA). Helium was used as the carrier gas with the GCMS set to a constant linear velocity of 46.8 cm/s. The injector was operated in splitless mode for 3 min

then switched to a 20:1 split ratio. The column oven was held at 50 °C for 3 min (during desorption of the SPME fiber), then heated to 240 °C at 10 °C/min then further increased to 250 °C at 30 °C/min and held at this temperature for 5 min. Total run time was 27.33 min. The interface and MS source temperatures were set at 250 °C and 200 °C. The MS was operated in electron impact (EI) mode at ionisation energy of 70 eV. All analytes were analysed in full scan mode.

2.3 Identification of FA methyl esters

Bacterial cells, 50 - 70 mg, were added to a 1.7 mL eppendorf tube followed by the addition of 0.1 mL of C13:0 tridecanoic acid 167 µg/mL (internal standard). These cells were then transferred to a glass tube (13 mm × 100 mm) with the aid of 1 mL of 3.75 N sodium hydroxide added to the eppendorf tube. The reaction conditions for both saponification and methylation reactions used were described by (Whittaker et al., 2005). The isolation of the fatty acid methyl esters (FAMES) was as follows; 3 mL of hexane was added before vortexing the tubes (5 min) with the lower phase then removed and 3 mL of 0.3 N sodium hydroxide added. Further vortexing of the tubes (5 min) was carried out before 0.5 mL of saturated sodium chloride was added (Sherlock MIS operating manual). To enhance separation of the organic and aqueous layers the tubes were centrifuged at 1,384 g for 5 min at room temperature. After this approximately 2.9 mL of the organic layer was transferred to a clean glass tube (13 mm × 100 mm) and evaporated to dryness with oxygen free nitrogen. The residue was then reconstituted in 50 µL of hexane, vortexed mixed, and transferred to a vial insert where it was further diluted with an additional 50 µL hexane used to rinse out the glass tube (Moss et al., 1974). The internal standard was prepared by dissolving 0.1001 g of Tridecanoic acid in 20 mL of methanol (HPLC grade) to make a primary standard. An aliquot of 0.334 mL of the primary standard was then added to a 10 mL volumetric flask and made up to the mark with methanol to make a final concentration of 167 µg/mL. A fatty acid methyl ester profile was then obtained using capillary GC column CP7420 100 m × 0.25 mm i.d. × 0.25 µm film thickness (Varian Column from Agilent Technologies s/n 6005241). This column was installed in a Shimadzu GC2010 gas chromatograph equipped with a flame ionisation detector (FID). The GC conditions were as follows; the injector was operated in split mode at a ratio of 15:1 with helium used as the carrier gas at a constant linear velocity of 16.7 cm/s, a sample volume of 1 µL was injected, the GC oven ramp (modified from that published by (Rugoho et al., 2014) was initially held at 45 °C for 4 min and then ramped to 175 °C at 13 °C/min held for 27 min and then further ramped to 215 °C at 4 °C/min and held for 35 min before a final ramp to 245 °C at 25 °C/min which was held for 10 min. The total run time was 97.2 min with both the injector and detector temperatures set at 250 °C. Fatty acid method esters were identified using retention time designations accompanying the Nuchek standard GLC 463. A Linear Retention Indices (LRI) were also calculated for each fatty acid methyl ester.

2.4 1D proteomics profile

Whole cell protein and cytosolic protein samples were prepared using a bead beating method (Hussain et al., 2009a) and the Pierce™ BCA Protein Assay Kit (Life Technologies, California, USA) was used to quantify the cytosolic proteins. 1D SDS PAGE was performed using a vertical slab system. The electrophoresis tank was filled with NuPAGE MOPS SDS Running Buffer. Then a commercial gel (NuPAGE ® Novex® Tris-Acetate Mini Gels) was inserted into the vertical slab of the electrophoresis unit. The high molecular weight protein marker and protein samples were loaded into the lanes of the gel. The lid of the electrophoresis tank was closed and electrophoresis was run at 70 V for 200 min or until the dye front reached the bottom of the gel sandwich. In order to visualise the protein bands, the gel after electrophoresis was placed in a staining ray containing coomassie brilliant blue stain overnight. After staining, the gel was washed a couple of times with RO water and then placed in a tray containing de-staining solution. The de-staining solution was replaced every two to three hours until the protein bands could be clearly visualised.

2.5 Data analysis

A general linear model was employed for the statistical analysis using the program Minitab 17 (Minitab Incorporation, USA) to evaluate the experimental data for VFA compounds production and FA profiles. The significant differences were accepted at $p < 0.05$ by Tukey's post hoc analysis for differences between treatments.

3. Results and Discussion

The GC-MS technique detected seven major VFA compounds in bacterial broth of low pH and bile salts stressed cells. The identified VFA compounds are listed in Table 1. Statistical analysis showed that the amount of acetic acid produced under standard and stress conditions were significantly different from each other. There was no significant difference between MI 13 and RC 2 under standard and stress conditions in the production of isobutyric acid. Butanoic acid, isovaleric acid and 2- methylbutanoic acid produced under

standard and low pH stress were significantly different from those produced under bile salts stress. Hexanoic acid and octanoic acid production was observed only in the case of bile salts stress conditions in MI 13 and RC 2.

Table 1: Volatile fatty acids detected in bacterial broth using HS- SPME and Shimadzu GC-MS QP-2010

Volatile fatty acid compounds	MI 13			RC 2		
	standard	pH stress	bile stress	standard	pH stress	bile stress
Acetic acid (mg/L)	4,225	2,192	3,375	4,411	2,371	3,091
Isobutyric acid (µg/L)	8,852	8,905	9,917	8,853	9,476	9,745
Butanoic acid (µg/L)	5,810	5,505	7,832	5,747	5,889	7,695
Isovaleric acid (µg/L)	3,424	3,138	4,881	3,423	3,412	4,801
2-methylbutanoic acid (µg/L)	1,235	1,099	1,719	1,221	1,178	1,696
Hexanoic acid (µg/L)	0	0	13,770	0	0	13,441
Octanoic acid (µg/L)	0	0	777	0	0	724

Identification of twenty four fatty acid methyl esters by GLC was observed when cells were exposed to standard and low pH and bile salts stress conditions. The different types of fatty acids produced by MI 13 and RC 2 under standard and low pH and bile stress conditions are shown in Figure 1. With MI 13, the SFA and MUFA levels were significantly different for all treatments. The PUFA levels for standard and low pH stress were the same, however increased for bile salts stress. With RC 2, SFA and MUFA levels were significantly different for all treatments. The PUFA levels for standard and low pH stress were the same but increased for bile salts stress. In general an increased level of PUFA production in both the isolates under bile salts stress was observed.

Seven different VFA compounds were produced by MI 13 and RC 2 under standard and stress conditions. However, their levels of production were different with each treatment. One key finding was the detection of hexanoic and octanoic acids which were produced only under the bile salts stress conditions. Although SFA, MUFA and PUFA were produced both under standard and stress conditions, their percentage levels varied with each treatment. Myristoleic, trans-palmitoleic, cis-palmitoleic, elaidic, trans-vaccenoic, petroselinic, oleic, cis- vaccenoic, gondoic and nervoic fatty acids (MUFA) showed the highest levels in all the three treatments, followed by tridecylic, teradecanoic, petadecylic, palmitic, margaric, nonadecyclic and stearic fatty acids (SFA). Linoleic, gamma- linolenic, linolenic and homo Ɣ linolenic fatty acids (PUFA) showed an increase under stress conditions, particularly bile salts. Previous reports suggest that the regulation of microbial cell membrane fatty acid profiles (Guerzoni et al., 2001) and maintenance of cell membrane functionality (Wu et al., 2012) are commonly known approaches employed by bacterial cells to combat environmental stresses such as low pH.

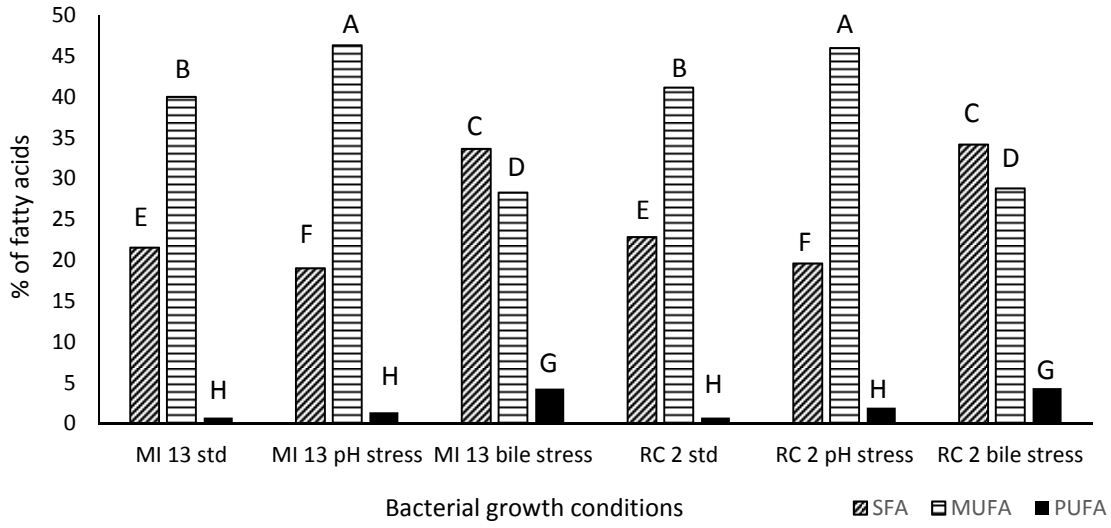

Figure 1: Types of fatty acid methyl esters produced by MI 13 and RC 2 under standard and pH and bile salts stress conditions. Columns with different letters differ significantly ($p < 0.05$)

Comparing the 1D protein banding patterns after pH and bile salts stress revealed a number of protein bands which were differentially expressed. With the whole cell proteomic profile, the most noticeable difference was the presence of dominant bands of MW ~40 kDa and ~78 kDa, which was seen after 16 h incubation with pH 3.5. It was visually difficult to detect any changes in protein expression banding patterns in case of bile salts stress with the whole cell profiles (data not shown). The cytosolic protein banding patterns were much more clear and distinct. Presence of dominant bands of MW ~32 kDa was observed after 16 h incubation with pH 3.5. Presence of dominant bands of MW ~32 kDa and ~30 kDa was observed after 16 h incubation with 3.5% bile salts, see Figure 2(a).

Unlike the dairy food isolate, clear distinguishable changes at pH 3.5 and 3.5 % bile salts after 16 h incubation were observed in the whole cell protein profile. The comparison of the protein profile banding patterns with pH stress and bile salts stress revealed a number of protein bands which were differentially expressed. With the whole cell proteomic profile, the most noticeable difference was the presence of dominant bands of MW ~78 kDa and ~110 kDa, which was seen after 16 h incubation with pH 3.5. With 3.5 % bile salts, the presence of dominant bands of MW ~78 kDa was observed after 16 h incubation, (data not shown). In case of cytosolic profiles, protein bands of MW ~110 kDa was observed after 16 h incubation with pH 3.5. Presence of dominant bands of MW ~80 kDa, ~100 kDa and ~160 kDa was observed after 16 h incubation with 3.5 % bile salts, see Figure 2(b).

The marked bands highlighted in lanes 4, 5, 6 and 7 showed changes in protein expression in Figure 2. Protein ladder marker (M); reference culture grown in standard MRS broth sampled after 24 h (lane 1); culture grown in standard MRS broth sampled after OD reaches 1.5 - 2 approximately 8 - 10 h (lane 2 and 3); culture grown in MRS broth of pH 3.5 (a) and pH 3.5 (b) sampled at 16 h (lane 4 and 5); culture grown in MRS broth containing 3.5 % bile salts (a) and 3.5 % bile salts (b) sampled at 16 h (lane 6 and 7).

From the 1D SDS PAGE protein profiles, dominant protein bands was observed with isolates MI 13 and RC 2 under pH 3.5 and 3.5 % bile salts stress after 16 h incubation. Isolate MI 13 displayed over-expressed bands following pH stress with whole cell protein profiles and bands were over- expressed with pH and bile salts stress for cytosolic profiles. Isolate RC 2 showed clear dominant bands following pH and bile salts stress for both whole cell and cytosolic protein profiles. The stress response can affect the synthesis of several proteins, more specifically stress proteins (Hussain et al., 2013). There are three different groups of stress proteins. (i) general stress proteins - the most commonly expressed proteins in response to most stresses and by likely all bacteria. They are induced non-specifically and are involved in DNA or protein repair. Examples include DnaK, GroEL, GroES or proteases like Clp proteases, (ii) specific stress proteins- proteins which are expressed under specific/particular stress conditions and (iii) proteins of general metabolism, that can be affected by some specific stresses (Champomier-Vergès et al., 2002) and conditions such as lactose starvation (Hussain et al., 2009a). 1D SDS PAGE technique is a useful to see an overview of the changes in the proteome but it does not provide information on the identity of the proteins. Further characterisation and identification of differential expressed proteins was not done in this study. It would be informative to identify important proteins that may serve as bacterial biomarkers for the selection of strains with the best probiotic potential.

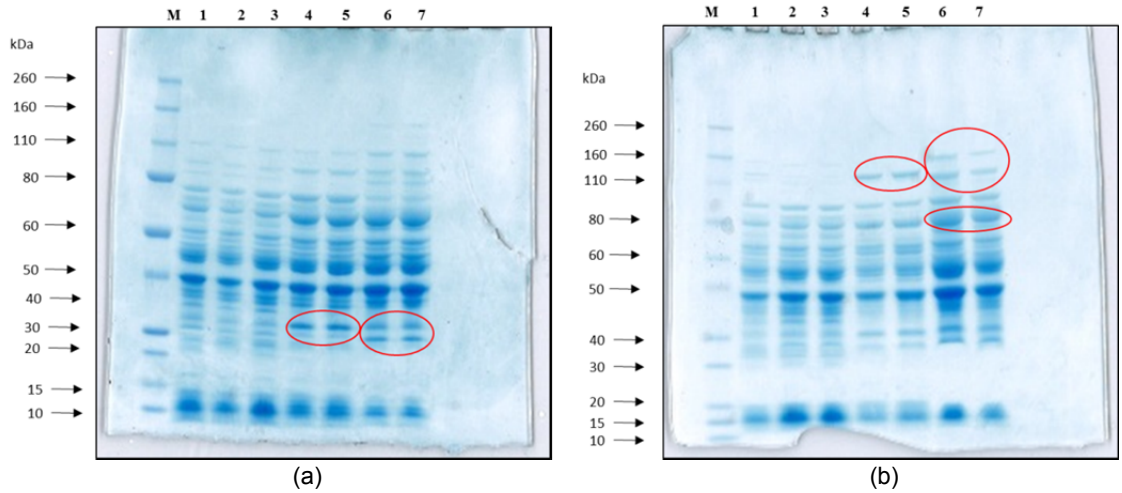

Figure 2: One-dimensional SDS-PAGE protein profile of cytosolic proteins from MI 13 (a) and RC 2 (b) cultures

4. Conclusion

Bacterial isolates compared in this study showed a great diversity in their biochemical characteristics. Significant differences were observed in the VFA production, bacterial FA profiles and ID banding pattern between bacterial species and stress conditions. Our results provide insight that may facilitate the development of new strategies for biotechnological applications of these species under physiological or environmental stress.

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