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LACTOBACILLUS FERMENTUM NCIMB 5221 HAS A GREATER FERULIC ACID PRODUCTION COMPARED TO OTHER FERULIC ACID ESTERASE PRODUCING LACTOBACILLI

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ABSTRACT: ***Ferulic acid (FA) is an antioxidant compound known to neutralize free radicals, such as reactive oxygen species (ROS). These free radicals have been shown to be involved in DNA damage, cancer and aging. The administration of FA, as an oral therapeutic is hampered by its absorption in the small intestine followed by its quick excretion. Colonic microbial enzymes have been shown to produce FA. In this article, selected Lactobacillus strains were screened for FA production by Ferulic Acid Esterase (FAE), as determined by the release of free FA from a natural substrate, ethyl ferulate (EFA). Using a MRS-EFA plate clearing assay, L. fermentum ATCC 11976, L. reuteri ATCC 23272 and L. fermentum NCIMB 5221 all showed clearance zones of 10mm in diameter, confirming FAE activity. Results show that L. fermentum NCIMB 5221 is the most efficient FA producing strain, producing 0.168±0.001mg/mL FA following 48 hours of incubation in 0.296mg/mL EFA. We also investigated the total antioxidant capacity of L. fermentum NCIMB 5221 when grown in culture. Results suggest that, due to its FA production, L. fermentum NCIMB 5221 has potential for use as a future therapeutic.***

KEY WORDS: Antioxidant, Bacteria, Ferulic acid, *Lactobacillus fermentum*, Probiotic

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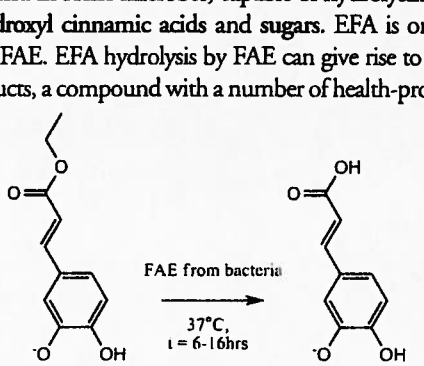
INTRODUCTION

Antioxidants are therapeutically interesting molecules capable of neutralizing free radicals, such as Reactive Oxygen Species (ROS). ROS have been implicated in DNA damage, cancer and accelerated cell aging (Lombard et al. 2005). Ferulic acid (FA), a phenolic acid, is a potent antioxidant able to neutralize these free radicals (Rice-Evans et al. 1996). Recent studies suggest that FA can have direct antitumor activity against breast cancer (Chang et al. 2006; Kampa et al. 2003) and liver cancer (Lee 2005; Taniguchi et al. 1999) and is also effective at preventing cancer induced by the exposure to various carcinogenic compounds such as benzopyrene (Lesca 1983) and 4-nitroquinoline 1-oxide (Tanaka et al. 1993). Along with its health beneficial properties, FA also exhibits a number of potential commercial applications in food preservation and in the production of vanillin, making it a compound of economic interest (Ou et al. 2004). Currently, FA is being produced industrially by batch fermentation using fungal ferulic acid esterases (FAE) (Bartolome et al. 1997; Bonnin et al. 1999). It has been proposed that gut bacterial cells also produce FAE, an enzyme that has the inherent capacity to produce FA from available substrates in the gastrointestinal tract Figure 1.

FIGURE 1. Ferulic acid esterases (FAE) are a subclass of cinnamoyl esterases, found in some microbes, capable of hydrolysing the ester bond between hydroxyl cinnamic acids and sugars. EFA is one of the natural substrates of FAE. EFA hydrolysis by FAE can give rise to FA as one of the desired products, a compound with a number of health-promoting benefits.



Ethyl ferulate Ferulic acid

In terms of a therapeutic, the oral delivery of free FA is limited due to its quick absorption in the small intestine, specifically in the jejunum, followed by its rapid excretion (Spencer et al. 1999; Zhao et al. 2003). The oral delivery of conjugated FA, in the form of a dietary source such as wheat bran, is a feasible alternative, with the release of free FA by microbial FAE present in the lower human digestive tract, giving rise to a constant and controlled release of FA (Spencer et al. 1999; Zhao et al. 2003). Attempts have also been made to develop probiotic therapeutic formulations to enhance the FA bioavailability for the treatment of inflammatory metabolic disorders (Bhathena et al. 2009). This article investigates Lactobacilli for FA production from a dietary substrate, explores the correlation between biomass production and FAE activity and investigates the antioxidant production of the most FAE-active strain.

Materials and Methods

Chemicals

Ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate, EFA) and ferulic acid (*trans*-4-hydroxy-3-methoxycinnamate, FA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). De Man, Rogosa, Sharpe (MRS) broth and Methanol of high-performance liquid chromatography (HPLC) grade were obtained from Fisher Scientific Canada (Ottawa, ON, Canada). Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). The QuantiChrom™ Antioxidant Assay Kit was purchased from BioAssay Systems (Hayward, California, USA). All other chemicals were of analytical or HPLC grade and purchased from commercial sources.

Bacterial strains and culture conditions

The bacterial strains *Lactobacillus reuteri* ATCC 23272, *Lactobacillus rhamnosus* ATCC 53103 and *Lactobacillus fermentum* ATCC 11976 were purchased from Cedarlane Laboratories (Burlington, ON, Canada). The bacterial strain *Lactobacillus fermentum* NCIMB 5221 was purchased from NCIMB (Aberdeen, Scotland, UK). All bacterial strains were stored at -80°C in MRS containing 20% (v/v) glycerol. An MRS-agar plate was streaked for isolation from the frozen stock and incubated at 37°C with 5% CO_2 for 24 hours to ensure purity. One colony from the MRS-agar plate was inoculated into 5mL of MRS broth and incubated at 37°C for 24 hours. A 1% (v/v) inoculum was then used for subculturing and incubated at 37°C for 24 hours immediately before use.

Ferulic acid esterase activity method: plate assay

Prior to the assay, the bacterial strains were subcultured from MRS broth at 1% (v/v) to MRS-EFA broth at an EFA concentration of 1.33mM and incubated at 37°C for 24 hours. MRS-EFA agar was prepared at 1.5% (w/v) of agar in MRS broth at pH 6.5 and autoclaved at 121°C for 15 minutes. The MRS agar was placed in a water bath to cool down to 55°C. 0.3mL of sterile EFA, prepared as a 10% (w/v) solution in dimethylformamide, was added per 20mL of agar mix. Using the poured and dried MRS-EFA agar plates, bacterial strains were impregnated, using three sterile Whatman #3 filter disks, in the culture from the MRS-EFA broth, placed on the MRS-EFA agar and then incubated at 37°C and 5% CO_2 for 48 hours. Each strain was impregnated on filter disks in triplicates to measure accuracy and reproducibility.

Ferulic acid esterase activity and FA production method: HPLC assay

All bacterial strains were subcultured from MRS broth at 1% (v/v) to MRS-EFA broth at an EFA concentration of 1.33mM (0.2956mg/mL). Uninoculated MRS-EFA broth was used as a negative control and treated in the exact same way. Each sample was treated in triplicate and incubated at 37°C during the course of the experiment. An HPLC assay, modified from Mastihuba et al., was used to measure FAE activity (Mastihuba et al. 2002). At every time point, 500µL of each sample was added to centrifuge tubes and centrifuged at 10,000rpm for 7 min at 4°C. The resulting supernatant (300µL) was acidified with 0.35M H_2SO_4 (100µL) and briefly vortexed. To the acidified solution, 1mM benzoic acid (300µL) was added, as an internal standard, to each tube followed by the addition of 0.7M NaOH (100µL) to neutralize the pH. The processed samples were then stored at -20°C prior to HPLC analysis.

For HPLC analysis, the samples were thawed to room temperature and filtered with a 0.45µm syringe filter. The HPLC analysis was performed on a reverse-phase C-18 column: LiChrosorb RP-18, 25 x 0.46cm (Grace Davison Discovery Sciences, ON, Canada). The HPLC system consists of a ProStar 335 diode array detector (DAD) set at 280nm and 320nm, a ProStar 410 autosampler, and the software Star LC workstation version 6.41. 25µL was injected for each sample. The mobile phase (solvent A) consisted of 37% (v/v) methanol and 0.9% (v/v) acetic acid in water (HPLC grade). Solvent B consisted of 100% (v/v) methanol. The HPLC run was initiated with solvent A at 100% for 16 minutes. This was then followed by a 1 minute linear gradient to reach 100% of solvent B, attained at the 17th minute. Solvent B was isocratically held at 100% for 12 minutes, until the 29th minute. This was then followed by a 1 minute linear gradient to reach 100% of solvent A by the 30th minute. Standard curves of FA and EFA, using peak area quantification, were generated for quantifying the test samples' FA and EFA concentrations. The FA standard curve was generated using triplicates and the concentrations 100, 300, 500, 960 and 1100µM were plotted against peak area (R^2=0.9869). The EFA standard curve was generated using triplicates and the concentrations 100, 300, 500, 700, 1000, 1400 and 1800µM were plotted against peak area (R^2=0.9785). Standards and quality control samples were prepared and analyzed in the exact same way as the test samples.

Method for correlating *L. fermentum* NCIMB 5221 growth with FAE activity

MRS-agar plates were streaked, inoculated and subcultured, as described above. A 1% (v/v) (350µL) inoculum was used for subculturing into 35mL of MRS broth and incubated at 37°C throughout the experiment. MRS-broth containing no inoculum was used as a negative control. At each time point, the culture tubes were shaken gently to ensure homogeneity and 200µL from each tube was pipetted into a 96-well plate and read using a UV spectrophotometer Victor^{3}V 1420 Multilabel Counter (Perkin Elmer, Boston, MA), at a wavelength of 620nm, with the readout correlated to a standard curve of absorbance vs. cell count. At each time point, samples were also prepared for a FAE HPLC assay, as described above. Each analysis was carried out in triplicate.

Total antioxidant capacity of *L. fermentum* NCIMB 5221

The total antioxidant production of *L. fermentum* NCIMB 5221 was measured using a QuantiChrom™ Antioxidant Assay Kit, a spectrophotometric assay based on the reduction of Cu^{2+} to Cu^{+}. The protocol provided with the assay kit was followed. A standard curve was generated for Trolox, a standard provided with the kit, at concentrations of 0, 300, 600 and 1000 µM and plotted against absorbance at 570nm (R^2=0.9970). A standard curve was also generated with FA at concentrations of 0.3, 0.6, 0.9, 1.2 and 1.5mM and plotted against absorbance at 570nm (R^2=0.9715). *L. fermentum* NCIMB 5221 was subcultured from MRS broth at 1% (v/v) to MRS-EFA broth at an EFA concentration of 1.5mM. Uninoculated MRS-EFA broth was used as a negative control. Each sample was treated in triplicate and incubated at 37°C during the course of the experiment. Samples were removed at every time point and were stored at -20°C until the assay was performed.

Statistical analysis

The experimental results are expressed as means ± Standard Deviation. Statistical analysis was carried out using Minitab (Minitab, Version 14, Minitab Inc, Pennsylvania, USA). Statistical comparisons between EFA/FA concentrations in media were carried out by using the general linear model (GLM). Statistical significance was set at $p < 0.05$. All interaction terms were treated as fixed terms and p-values less than 0.01 were considered highly significant.

Results

Ferulic acid esterase activity by a plate assay

Ethyl ferulate (EFA) clearing zones, due to FAE hydrolysis, were visible for *L. fermentum* ATCC 11976 and *L. reuteri* ATCC 23272 (positive controls), but also for the test strain, *L. fermentum* NCIMB 5221 Figure 2. The zones of clearance were 10 mm in diameter with no significant difference between the strains. The negative control strain, *L. rhamnosus* ATCC 53103, and the unimpregnated disk showed no visible clearance on the MRS-EFA agar.

FIGURE 2. Clearing zones on MRS-EFA agar indicate the presence of FAE activity. A) MRS-EFA agar with no bacteria; B) MRS-EFA agar with *L. rhamnosus* ATCC 53103; C) MRS agar with *L. fermentum* ATCC 11976; D) MRS-EFA agar with *L. fermentum* ATCC 11976; E) MRS-EFA agar with *L. reuteri* ATCC 23272; F) MRS-EFA agar with *L. fermentum* NCIMB 5221. The zones of clearance are 10mm in diameter, with no significant difference between positive strains.

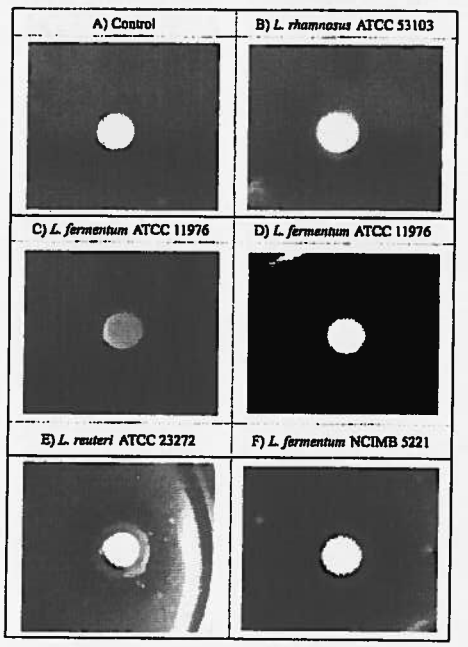
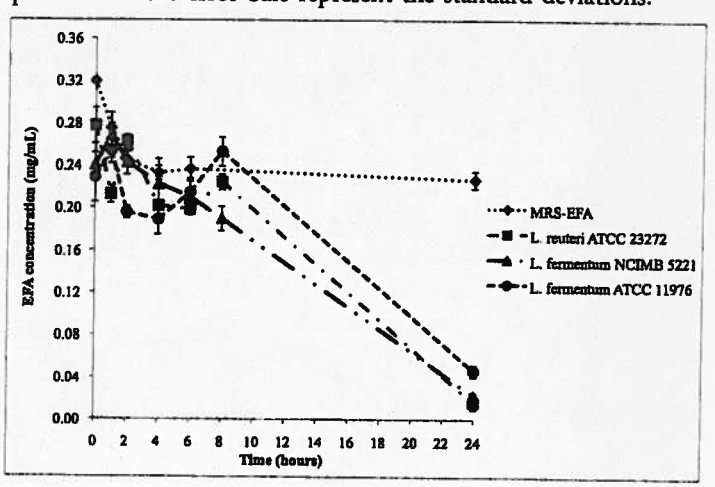


FIGURE 3. FAE quantitative HPLC assay for *L. fermentum* ATCC 11976, *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221. Uninoculated MRS-EFA was used as a negative control. The data represents the amount of EFA, the FAE substrate, remaining in solution, as measured by HPLC peak area data converted to EFA concentration values using a standard curve for EFA concentration vs. peak area. The initial concentration of MRS-EFA was 1.33mM (0.2956mg/mL). Following 24 hours of incubation, *L. reuteri* ATCC 23272 (0.01625±0.00673mg/mL) and *L. fermentum* NCIMB 5221 (0.02297±0.00232mg/mL) had the lowest concentrations of unhydrolysed EFA when compared to *L. fermentum* ATCC 11976 (0.04611±0.00561mg/mL). Each point represents the mean of triplicates and the error bars represent the standard deviations.



Ferulic acid esterase activity and FA production by HPLC

MRS-EFA at a concentration of 1.33mM (0.2956 mg/mL) was used. Following 24 hours of incubation, *L. reuteri* ATCC 23272 (0.01625±0.00673 mg/mL) and *L. fermentum* NCIMB 5221 (0.02297±0.00232 mg/mL) had the lowest concentrations of unhydrolysed EFA remaining in solution when compared to *L. fermentum* ATCC 11976 (0.04611±0.00561 mg/mL) Figure 3. Based on these results, *L. fermentum* NCIMB 5221 and *L. reuteri* ATCC 23272 were chosen for further FAE activity characterisation by HPLC.

The HPLC screening method was repeated, with only the two strains aforementioned. The hydrolysis of EFA by *L. fermentum* NCIMB 5221 and *L. reuteri* ATCC 23272 followed a similar pattern for both bacterial strains, through the 48 hours of the assay. Following 48 hours of incubation, *L. reuteri* ATCC 23272 was observed to have a lower concentration of unhydrolysed EFA in the solution at 0.00358±0.00030 mg/mL, when compared to *L. fermentum* NCIMB 5221 with 0.03917±0.00320 mg/mL remaining Figure 4a. FA quantification was performed for a direct measurement of the desired end-product of FAE activity. Surprisingly, FA quantification demonstrated the absence of any FA production by *L. reuteri* ATCC 23272, despite its efficient EFA hydrolysis Figure 4b. *L. fermentum* NCIMB 5221 had a final production, following 48 hours, of 0.16847±0.00122 mg/mL FA. The summary of the results, following 24 hours of incubation of the strains in MRS-EFA, is presented in Table 1.

FIGURE 4. FAE quantitative HPLC assay for *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221. Uninoculated MRS-EFA was used as a negative control. The presented data represents the amount of unhydrolysed EFA remaining in solution (A) and the amount of FA produced (B), as measured by HPLC peak area data converted to EFA and FA concentration values using standard curves for EFA and FA concentration vs. peak area. The initial concentration of MRS-EFA was 1.33mM (0.2956mg/mL). (A) Following 48 hours of incubation, *L. reuteri* ATCC 23272 had 0.00358±0.00030mg/mL and *L. fermentum* NCIMB 5221 had 0.03917±0.00320mg/mL unhydrolysed EFA. (B) FA quantification demonstrates the absence of any FA production by *L. reuteri* ATCC 23272 throughout the experiment. *L. fermentum* NCIMB 5221 had a final production, following 48 hours, of 0.16847±0.00122mg/mL FA. Each point represents the mean of triplicates and the error bars represent the standard deviations.

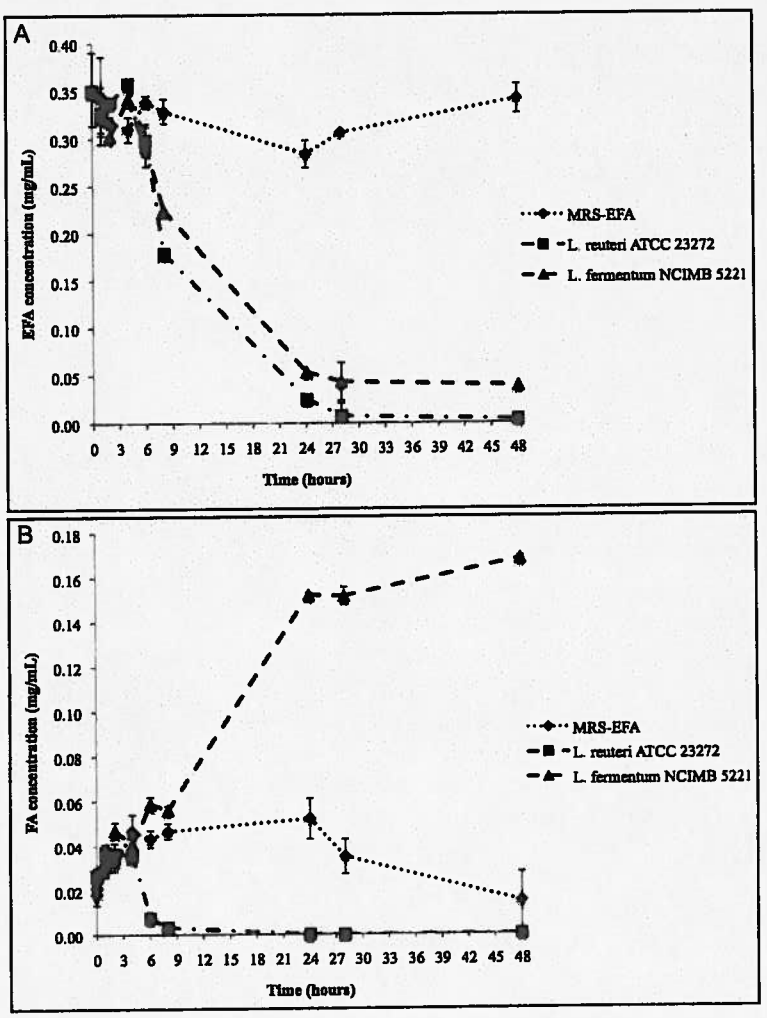


TABLE 1. A comparison of EFA hydrolysis and FA production for *L. fermentum* NCIMB 5221, *L. reuteri* ATCC 23272 and *L. fermentum* ATCC 11976, following 24 hours of incubation in MRS-EFA (0.2956mg/mL EFA).The data represents the amount of unhydrolysed EFA, also expressed as efficacy of EFA hydrolysis and FA production. FA quantification demonstrates the absence of any FA production by *L. reuteri* ATCC 23272 throughout the experiment. *L. fermentum* ATCC 11976 (results not shown in the HPLC graphs), had a production of 0.10348±0.00442mg/mL FA. *L. fermentum* NCIMB 5221 had a production of 0.15221±0.00101mg/mL FA.The data values represent the mean of triplicates with the standard deviation associated with the measurements.

Bacterial strain	24 hr EFA(mg/mL)	24 hr EFA hydrolysis(%)	24 hr FA production(mg/mL)
L. fermentum ATCC 11976	0.04611±0.00561	84.4±1.9	0.10348±0.00442
L. reuteri ATCC 23272	0.01625±0.00673	94.5±2.3	Not detectable
L. fermentum NCIMB 5221	0.02297±0.00232	92.2±0.8	0.15221±0.00101

Correlating *L. fermentum* NCIMB 5221 growth with FAE activity

To investigate the FAE enzyme kinetics, with relation to *L. fermentum* NCIMB 5221's growth kinetics, growth and FA production were investigated in parallel. Results in Figure 5a show a lag phase between 0 and 6 hours, the exponential phase from 6 hours ($3.27x10^8$ cfu/mL) to 16 hours ($1.32x10^9$ cfu/mL) and the stationary phase starting at 16 hours. The correlated FA production curve, generated by an HPLC assay, elucidates the direct proportionality between bacterial cell growth and FAE enzyme kinetics Figure 5b. The higher the growth rate of *L. fermentum* NCIMB 5221, exemplified by the logarithmic scale of its growth curve, the greater was its rate of FA production.

Total antioxidant capacity of *L. fermentum* NCIMB 5221

To investigate the antioxidative potential of the FA produced by *L. fermentum* NCIMB 5221, the strain was incubated in MRS-EFA at an initial concentration of 1.5mM and supernatant was collected at various time points. The supernatant samples were measured using an antioxidant measurement kit. Following 48 hours of incubation, *L. fermentum* NCIMB 5221 had a production of 509.58±13.23 µM Trolox, equivalent to 857.16±22.25µM FA Figure 6. It can also be concluded that the levels of antioxidant compounds increased for the first 16 hours and then reached a plateau. This is the same trend observed with the HPLC results aforementioned.

FIGURE 5. The growth curve (A) of *L. fermentum* NCIMB 5221 correlated with FA production (B). (A) The growth curve was generated by absorbance at 620nm over time and correlation to a standard curve of absorbance vs. colony forming units. The growth curve is characterized by the lag phase between 0 and 6 hours, the exponential phase between 6 ($3.27x10^8$ cfu/mL) and 16 hours ($1.32x10^9$ cfu/mL) and the stationary phase from 16 hours on. (B) The correlated FA production data represents the amount of FA produced, as measured by HPLC peak area data converted to FA concentration values using a standard curve for FA concentration vs. peak area. The initial concentration of MRS-EFA was 1.33mM (0.2956mg/mL). These results elucidate the direct relationship between bacterial cell growth and enzyme kinetics.The FA production rate is maximal during the exponential phase of growth of *L. fermentum* NCIMB 5221. Each point represents the mean of triplicates and the error bars represent the standard deviations.

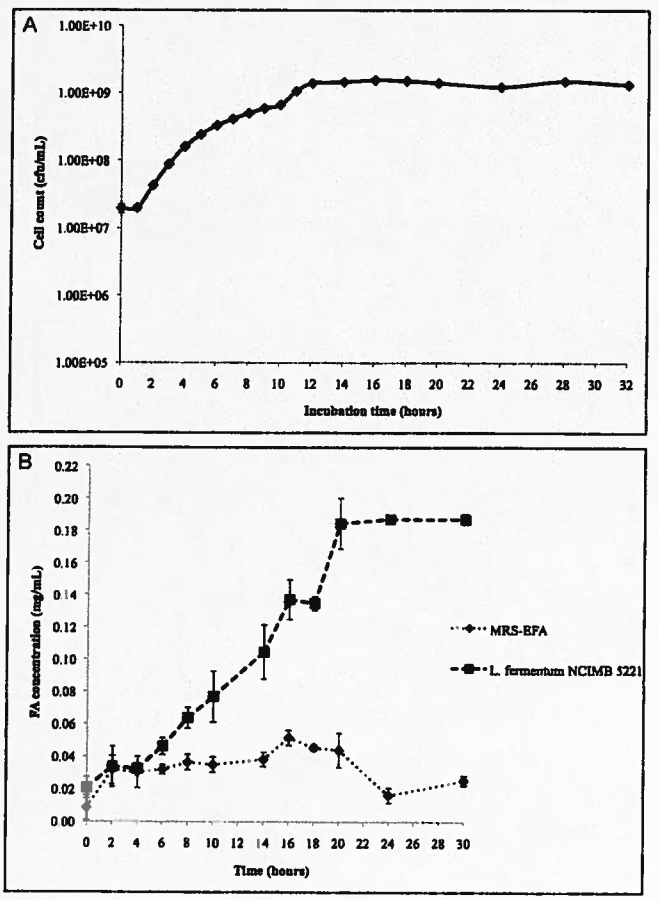
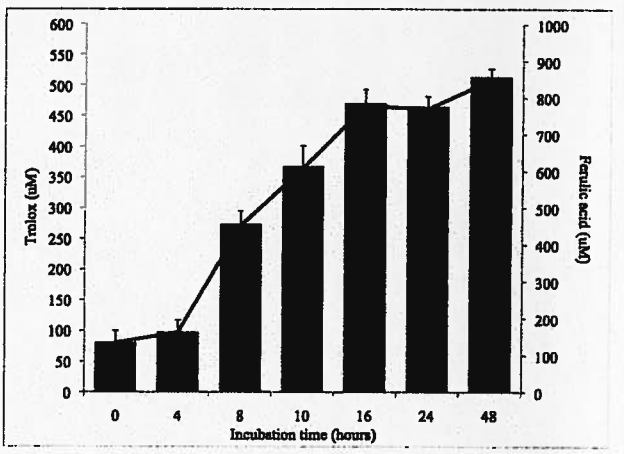


FIGURE 6. Measurement of total antioxidant production by *L. fermentum* NCIMB 5221 incubated in an initial concentration of MRS-EFA of 1.5mM. Standard curves for Trolox and FA were generated and used to determine antioxidant concentration. Following 48 hours of incubation, *L. fermentum* NCIMB 5221 had a production of 509.58±13.23 µM Trolox, equivalent to 857.16±22.25 µM FA. Each point represents the mean of triplicates and the error bars represent the standard deviations



Discussion

Probiotics are dietary supplements containing bacteria which, when administered in adequate amounts, confer a health benefit on the host (FAO et al. 2001). Due to the proven safety of many bacterial strains present in current probiotic formulations, their clinical applications have gained wide interest. Various bacterial strains have been shown to be beneficial in a wide range of conditions including infections, allergies and metabolic disorders such as ulcerative colitis and Crohn's disease (Al-Salami et al. 2008a; Floch 2010; Jankovic et al. 2010; Luoto et al. 2010; Wolvers et al. 2010). The most common microorganisms used in probiotic formulations are the lactic acid bacteria (LAB), which are important components of the healthy gut microbiota and have been regarded as safe by the American FDA (Parvez et al. 2006). Lactobacilli have

shown unique advantages in metabolic diseases through the production of useful enzymes that exert desirable biological activities in the host (Al-Salami et al. 2008b; Guglielmetti et al. 2008). Current research focuses on using specific bacterial strains that secrete or produce useful biologically active enzymes and proteins (Azcarate-Peril et al. 2004; Guglielmetti et al. 2008). These enzymes include ornithine decarboxylase, used as a potent antioxidant for the treatment of oxidative stress associated autoimmune diseases and accelerated cell apoptosis (Mates et al. 2002), bile salt hydrolase for hypercholesterolemia and cholesterolosis (Martoni et al. 2007; Martoni et al. 2008; Tanaka et al. 1999), and bile transport and tolerance proteins for efficient delivery of probiotics (Pfeiler et al. 2009). Another type of enzyme that recently gained interest in food production is cinnamoyl esterases (Guglielmetti et al. 2008). In recent studies, the products of cinnamoyl esterase have shown remarkably high levels of antioxidant activity (Srinivasan et al. 2007) and other health-promoting effects, including stimulation of insulin secretion (Adisakwattana et al. 2008; Sri Balasubashini et al. 2003), prevention of oxidative stress (Srinivasan et al. 2007), lipid peroxidation (Balasubashini et al. 2004), cholesterol lowering capabilities (Bhathena et al. 2009) and inhibition of diabetic nephrophathy progression (Atsuyo et al. 2008). Cinnamoyl esterase enzymatic activities include the production of caffeic, ferulic, sinapic and *p*-coumaric acids (Fazary et al. 2007). FAE are a subclass of cinnamoyl esterases, found mainly in plants, but also in some microbes, capable of hydrolysing the ester bond between hydroxyl cinnamic acids and sugars (Crepin et al. 2004). FA, a well-characterised antioxidant, is one of the desired products of hydrolysis by FAE. Gut bacteria have been described as having FAE activity, with probiotic bacteria already demonstrating health beneficial properties through FA production (Bhathena et al. 2009). In this research, we focus on probiotic Lactobacilli, specifically investigating the strains for inherent FA production.

In recently published articles, the FAE precipitation plate assay was used to screen for FAE and a strong activity was shown by *L. fermentum* ATCC 11976 and *L. reuteri* ATCC 23272 (Bhathena et al. 2007). The plate screening assay was performed with *L. fermentum* ATCC 11976 and *L. reuteri* ATCC 23272 as positive controls, with *L. rhamnosus* ATCC 53103 as a negative control and with *L. fermentum* NCIMB 5221 as the strain to be screened for FAE activity. As expected, EFA clearing zones, due to FAE activity, were obtained for the positive control strains and no clearing zone was observed for the negative control strain. *L. fermentum* NCIMB 5221 tested positive for FAE activity, with a clearing size approximately equivalent to the two positive control strains. The EFA plate assay remains qualitative, so further investigation into the strains' FAE activity was necessary.

An HPLC assay was used for the quantification of the FAE activity, in terms of EFA hydrolysis, of *L. fermentum* ATCC 11976, *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221. Following 24 hours of incubation, *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221 had the lowest concentrations of unhydrolysed EFA in comparison *to L. fermentum* ATCC 11976. *L. reuteri* ATCC 23272 had a slightly lesser concentration of unhydrolysed EFA than did *L. fermentum* NCIMB 5221. For further investigation into the different FAE activity profiles of *L. reuteri* ATCC 23272 and *L. fermentum* NCIMB 5221, quantification was performed for EFA hydrolysis and FA production, in parallel. Following 48 hours of incubation, *L. reuteri* ATCC 23272, as the previous assay results, was observed to have a lower concentration of unhydrolysed EFA than *L. fermentum* NCIMB 5221. Unexpectedly, the quantification of FA production by *L. reuteri* ATCC 23272 demonstrated the absence of any FA, even lower than the background values of the control. This suggests enzymatic activity of *L. reuteri* ATCC 23272 which can use FA as a precursor for the production of other compounds. It has been shown that, through microbial transformation, vanillic acid can be produced from FA, a conversion that occurs by the removal of the phenyl-propenoic C_2-side chain from FA (Bloem et al. 2007; Ghosh et al. 2007). Vanillic acid has, as well, been shown to have antioxidant activity, although not as effective as that of FA (Baublis et al. 2000). Hence, *L. fermentum* NCIMB 5221 was used for further investigations, although further work is required to characterize the end-product of *L. reuteri* ATCC 23272 metabolism of EFA.

There was a need to further characterize *L. fermentum* NCIMB 5221 in terms of its growth kinetics with relation to FAE activity. It was demonstrated that the FA production rate is maximum between 6 and 16 hours, correlating with the exponential phase of *L. fermentum* NCIMB 5221. This is important for future studies, *in vitro* and *in vivo*, in terms of timing bacterial cell cultivation at the appropriate stage of culture growth. In the case of *L. fermentum* NCIMB 5221, the bacterial cells should be cultivated at the point of highest cell turbidity, at the late exponential or early stationary phase, where enzyme activity is also greatest.

Investigations into the antioxidant potential of FA produced by *L. fermentum* NCIMB 5221 was also performed using an antioxidant kit which measures the total antioxidant potential present in solutions, based on the reduction of Cu^{2+} to Cu^{+}. It was clearly demonstrated that, when incubated with EFA, an FAE substrate, *L. fermentum* NCIMB 5221 produced a significant level of antioxidants, correlating with the levels of FA quantified using the HPLC methods. These results prove the functionality of the FA molecule rather than just its presence in solution.

Future work should involve additional characterisation of a final formulation for potential preclinical use, in terms of the mechanisms of action and safety of the probiotic strain and the produced FA with *in vitro* and *in vivo* studies. FAE activity also needs to be tested using more complex substrates than EFA, such as wheat bran, to more closely resemble the dietary intake of FA precursors. The successful delivery of bacteria through the upper gastrointestinal tract remains an

issue, and this strain needs to be investigated for characteristics such as bile resistance. Techniques such as microencapsulation may also prove useful for efficient delivery to the lower gastrointestinal tract, the desired site of action of probiotics (Bhathena et al. 2009; Chen et al. 2010; Urbanska et al. 2009). In terms of the gastrointestinal tract, FAE activity should be characterized in terms of FA production in a gastrointestinal system and *in vivo* in appropriate animal models to ensure enzyme activity remains stable and efficient under potentially harsh conditions. There still remains a great deal of work to be done before a FAE active bacterial strain, such as *L. fermentum* NCIMB 5221, can be developed into an efficient therapeutic.

In summary, the presented work successfully screened for FAE active bacteria using a qualitative precipitation assay on MRS-EFA agar and a quantitative HPLC assay. The most active FAE bacterial strain in terms of FA production, from the selected bacteria, was shown to be *L. fermentum* NCIMB 5221. The FAE activity of this strain was correlated with its growth curve, demonstrating a direct relationship between bacterial cell count and FA production. The total antioxidant capacity of the strain was also investigated. This work opens up future potentials for using a synergistic formulation of *L. fermentum* NCIMB 5221 with its intrinsic microbial FAE activity for both industrial and therapeutic applications.

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