An *in vitro* antimicrobial and safety study of *Lactobacillus reuteri* DPC16 for validation of probiotic concept

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Abstract

Based on previous studies of the novel *Lactobacillus reuteri* DPC16 strain, an *in vitro* investigation on the supernatant antimicrobial activity and the culture safety against normal gastrointestinal microflora and gastric mucus was done in this thesis.

DPC16 cell-free supernatants (fresh and freeze-dried, designated as MRSc and FZMRSc) from anaerobic incubations in pre-reduced MRS broth, have shown significant inhibitory effects against selected pathogens, including *Salmonella* Typhimurium, *E. coli* O157:H7, *Staphylococcus aureus*, and *Listeria monocytogenes*. These effects were mainly due to the acid production during incubation as evidenced by the negation of such activity from their pH-neutral counterparts, and this acidic effect was shown to reduce the pathogen growth rate and decrease the total number of pathogen cells.

By incubation of concentrated (11 g/L) resting cells in glycerol-supplemented MRS broth, another DPC16 cell-free supernatant (designated as MRSg) has shown very strong antimicrobial effect against all target pathogens. As indicated by a kinetic profile, this activity developed in a sigmoidal fashion as incubation proceeded, reaching to maximum activity after 6-8h and maintained at the same level thereafter. Further study has shown that the antimicrobial activity of this supernatant was pH-independent, effective across a pH range of 4.6 to 6.5, and acted on both Gram-negative and Gram-positive pathogens. Using the minimum effective dose, a time course investigation has provided evidence that this supernatant affected the growth of the target pathogens by elongating the lag phase and lowering the total cell number at the end of the incubation. Lastly, it was found that the strong antimicrobial effect of MRSg was bactericidal at high concentrations and bacteriostatic at low concentrations. However, it also found that the viability of DPC16 cells also decreased as incubation prolonged, which suggests that this glycerol-derived supernatant had a lethal effect to its own cells. Nevertheless, this lethal effect was exerted to a much lesser extent compared with that to the pathogens.

The last DPC16 cell-free supernatant was designated as SGF, which was produced from secondary fermentation of the same resting cells in glycerol-water. SGF did not show a

significant antimicrobial activity, which suggests that this specific strain is not capable of utilising glycerol in the absence of fermentable carbohydrates.

The antimicrobial activity found in MRSg matched with previously published characteristics of reuterin, which is a unique antimicrobial substance synthesised by *L. reuteri* when incubated with glycerol. Therefore, a study on the production kinetics of reuterin by DPC16 was carried out. Supernatants of both MRSg and SGF were studied. Results showed that glycerol utilisation occurred in both MRSg and SGF, whereas the bioconversion of glycerol into reuterin was different. In MRSg, glycerol was constantly utilised by DPC16 resting cells, and by the end of an 18h incubation 85.8 mM of glycerol was utilised, where 72.8% was transformed into reuterin. The formation of reuterin initiated with an inclining production and reached the maximum rate of 10.9 mM/h after 6h of incubation, with the total production of 64 mM of reuterin at the end of the 18h incubation. This reuterin production in MRSg followed a similar pattern to that of its antimicrobial activity, which suggests a certain correlation between reuterin formation and the increase of antimicrobial activity in MRSg. Therefore, the major antimicrobial component in MRSg that accounted for its potent antimicrobial activity was very much likely to be reuterin. In SGF, however, detectable reuterin was negligible even though some glycerol may have been absorbed into the highly concentrated DPC16 resting cells. This has responded to the antimicrobial activity assay in that due to the lack of essential carbohydrate nutrient for normal cell metabolism, there was no glycerol utilisation and hence no reuterin synthesis.

Having studied the antimicrobial activity of *L. reuteri* DPC16 and the production of antimicrobial-competent reuterin, two safety issues (the impact on growth on other normal commensal probiotics and mucin degradation activity) of this strain were assessed to further evaluate its probiotic potential. By using similar *in vitro* assays as in the antimicrobial test, the same set of DPC16 supernatants have demonstrated no adverse effect on the growth of either *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, or *Bifidobacterium lactis* DR10. No stimulatory effect was found either. By incorporating purified porcine gastric mucin into classic mucin-degradation assays in both liquid and agar media, DPC16 has not exhibited the same mucinolytic activity as that of the faecal flora cultures. Thus, it can be concluded that *L. reuteri* DPC16 is as safe to the host as normal commensal microflora.

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Abbreviations

3-HPA	3-hydroxypropionaldehyde
BbL	*B. lactis* DR10 culture
BHI	Brain heart infusion broth
EMP	Embden-Meyerhof pathway
FF	Faecal flora culture
FHEF	Facultative hetero-fermentation
FZMRSc	Aqueous solution of freeze-dried MRSc
GI	Gastrointestinal
HFF	Heat-killed faecal flora culture
LAB	Lactic acid bacteria
LR	*L. reuteri* DPC16 culture in pre-reduced MRS
LRg	*L. reuteri DPC16* glycerol fermentation
MAP	Modified atmosphere packaging
MIC	Minimum inhibition concentration
MRSc	DPC16 supernatant from incubation in MRS
MRSg	DPC16 supernatant derived from glycerol-MRS incubation
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD+
OD	Optical density
OHEF	Obligate hetero-fermentation
OHOF	Obligate homo-fermentation
PBS	Phosphate buffered saline
PKP	Phosphoketolase pathway
PGM	Porcine gastric mucin
RPM	Revolution per minute
SCFA	Short chain fatty acids
SEM	Standard error of the mean
SGF	Sole glycerol ferment (DPC16 supernatant from glycerol-water fermentation)

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