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IN-VITRO GROWTH CHARACTERISTICS OF COMMERCIAL PROBIOTIC STRAINS AND THEIR POTENTIAL FOR INHIBITION OF *CLOSTRIDIUM DIFFICILE* AND *CLOSTRIDIDUM PERFRINGENS*

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Background

C. difficile and *C. perfringens* are important animal pathogens. There are currently no preventative measures and therapeutics are limited to antibiotics. With antibiotic resistance on the rise new approaches are needed.

Probiotics have been evaluated in humans in relation to clostridial disease and results have been promising. There are no animal probiotics licensed for use against clostridial disease.

Many strains that show promising activity can not be commercially produced as they are not technically robust. This could be overcome by using strains already in commercial production.

Objective and Hypothesis

1)To assess the ability of selected commercial probiotic strains to inhibit growth of *C. difficile* and/or *C. perfringens* in vitro

2)To evaluate their ability to grow in the presence of oxygen, acid and bile

Strain	Trade Name	MRS	pH 4	pH 2	Bile 0.3%	Bile 0.15%
L. plantarum	BG112	100	81	1	85	95
L. rhamnosus	LRH19	100	40	0	63	83
L. plantarum	LPAL	100	60	1	90	99
L. rhamnosus	SP1	100	75	0	71	81
B. animalis spp lactis	BLC1	100	77	0	92	96
		Growth in % compared to MRS				

Table 2 Growth characteristics of 5 selected probiotic strains with inhibitory activity against clostridia in standard Man-Rogosa-Sharp (MRS) broth, MRS broth adjusted to pH2.0 and pH4.0, and MRS broth with 0.3% or 0.15% bile added. The numbers are given as percentage of growth compared to growth in normal MRS broth

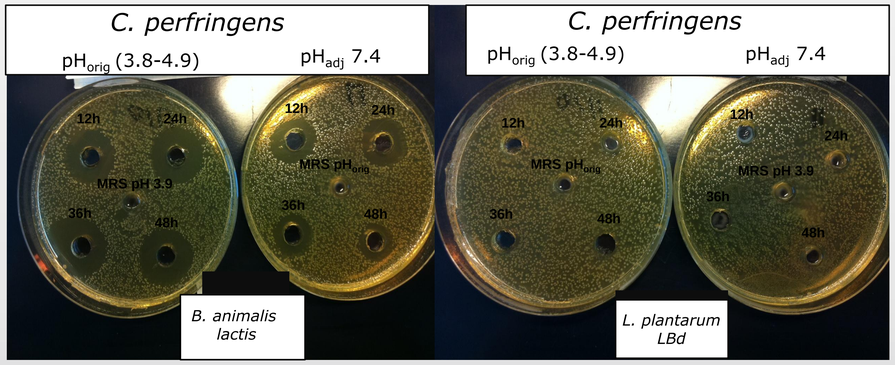

Figure 1 Inhibition of *C. perfringens* by cell-free supernatants at pH$_{orig}$ and pH$_{adj}$, obtained from *B. animalis lactis BLC1* and unsuccessful inhibition by *L. plantarum LBd*.
12h, 24h, 36h, 48h: Probiotic supernatant obtained after respective incubation time.
Center well: Sterile MRS broth adjusted to pH3.9 or standard MRS broth (pH$_{orig}$)

Material and Methods

Seventeen probiotic strains were used (Table 1).

Inhibition of C. difficile and C. perfringens

The effect of a cell free probiotic culture supernatant on the growth of *C. difficile* ribotype 078 and *C. perfringens* Type C was assessed. Supernatant was harvested and sterilized after 12, 24, 36, 48, and 72 hours and six days. One aliquot was adjusted to pH7.4 (pH$_{adj}$) the other aliquot was left at original pH (pH$_{orig}$).

Agar well diffusion assay

The anti-clostridial activity was evaluated by agar well diffusion following addition of supernatant at pH$_{orig}$ or pH$_{adj}$.

Broth co-culture in Brain Heart Infusion (BHI)

BHI broth was inoculated with *C. difficile* or *C. perfringens* and probiotic supernatant (t48h, pH$_{orig}$ or pH$_{adj}$). Clostridial growth was compared to growth of a control culture with Man-Rogosa-Sharpe (MRS) at pH7.0 or pH3.9 instead of supernatant using spectophotometry. Inhibition was indicated by a reduction of growth of at least 50%.

Evaluation of growth characteristics

Aerobic and anaerobic growth of probiotics was assessed visually. Growth was compared between growth in standard MRS broth, MRS pH2.0 and pH4.0, and MRS supplemented with 0.15% and 0.3% bile spectophotometrically.

Results

Inhibition of C. difficile and C. perfringens

Agar well diffusion assay (Fig. 1)

- 2/17 strains inhibited *C. perfringens* (Tab. 1)
  - Supernatant from all timepoints (growth phases) was inhibitory
  - Supernatant at pH$_{orig}$ and pH$_{adj}$ was inhibitory
  - Inhibition of pH$_{orig}$ was greater indicating presence of an additional antibiotic compound other than organic acids
- 10/ 12 strains inhibited *C. difficile* (Tab. 1)
  - Only supernatant with pH$_{orig}$ was inhibitory indicating inhibition due to organic acids
  - Only supernatant harvested after at least 36h of incubation inhibited *C. difficile*.

Broth co-culture

- 5/17 probiotics inhibited *C. perfringens* and 10/17 inhibited *C. difficile* (Tab. 1)
- Inhibition was only seen with supernatant at pH$_{orig}$

Growth characteristics

- All strains grew aerobically except *B. animalis lactis*.
- None of the strains grew at pH2, growth at pH4 ranged between 40-95%
- Growth ranged from 40-99% when bile was added
- Growth parameters of selected probiotic strains that showed inhibitory potential against both clostridia are presented in Table 2.

Strain	Trade Name	*C. difficile* Agar Well	*C. difficile* Broth	*C. perfringens* Agar Well	*C. perfringens* Broth
L. plantarum	BG112	√	√	x	√
E. faecium	EF1	x	x	x	x
L. casei	LC11	√	√	x	x
L. rhamnosus	LRH19	√	√	x	√
L. fermentum	Lba	x	x	x	x
L. helveticus	LH62	x	x	x	x
L. brevis	LBR9	x	x	x	x
L. salivarius	LBb	√	x	x	x
L. plantarum	LBc	√	√	x	x
L. plantarum	LPAL	√	√	√	√
L. plantarum	LBd	√	√	x	x
L. casei	LC10	x	√	x	x
L. sakei	LS-B	x	x	x	x
L. rhamnosus	LRB	√	√	x	x
E. faecium	SF02	x	x	x	x
L. rhamnosus	SP1	√	√	x	√
B. animalis spp lactis	BLC1	√	√	√	√

Table 1 Inhibitory activity of probiotic supernatant against *C. perfringens* and *C. difficile* in agar well diffusion and broth co-culture assays. Probiotics outlined in yellow and blue showed inhibition against both clostridia. Probiotics outlined in blue showed inhibition against both clostridia in both assays. Fields outlined in green show inhibitory potential in the respective experiment against the respective clostridial strain.

Conclusions

• 5 strains (*L. plantarum (n=2), L. rhamnosus (n=2)* and *B. animalis lactis)* inhibit clostridial growth by a reduction of pH. This inhibitory effect is likely due to organic acid production during stationary growth phase

• 2 of the 5 strains (*L. plantarum, B. animalis)* produce an additional antibiotic compound that inhibits *C. perfringens* only. This compound is produced during the exponential phase and it's activity is pH-independent.

•These 5 strains show growth characteristics suitable for probiotics and their possible use for control of clostridial disease will be further explored by in-vitro and in-vivo studies.

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Declaration of Conflict of Interest: One of the co-authors (P Dedenroth) is employed by the Clerici-Sacco Group (Cadorago, Italy), the company who supplied the probiotic strains for this study. The company did not make a financial contribution to this study and had no influence on study design or reporting of results.