CHAPTER 3

RESULTS

3.1 Effects of *E. faecalis* supernatant on MMP-2 expression and activation in cultured fibroblasts from human periodontal ligament

3.1.1 Cytotoxicity of *E. faecalis* supernatant on cultured fibroblasts : MTT assay

Fibroblasts were cultured in the presence of *E. faecalis* supernatant, either ATCC 19433 or ATCC 14506 at various concentrations (2-40% (v/v)) for 24 hours. The toxicity of the supernatant was tested by MTT assay. The results, as shown in Table 3.1, revealed that ATCC 19433 and ATCC 14506 *E. faecalis* supernatant were found non toxic at ≤ 15% (v/v). No significant difference in viable cell numbers between any of the cells treated at the concentrations of 2, 10, and 15% (v/v) and the control groups ($p > 0.05$). However, the number of viable cells decreased significantly at higher concentrations [20% (v/v) onward], with an indication of *E. faecalis* supernatant toxicity. Therefore, 2, 10, and 15% (v/v) of *E. faecalis* supernatant were chosen for further experiment.

3.1.2 Regulation of MMP-2 expression and activation in cultured fibroblasts by *E. faecalis* supernatant : Gelatin zymography

Fibroblasts were cultured in the presence of 2%, 10% and 15% (v/v) of *E. faecalis* supernatant, either ATCC 19433 or ATCC 14506, for 48 hours. Cultured fibroblasts stimulated with Con A, 25 µg/ml, were used as the positive

control, whereas cultured fibroblasts in the presence of 15% (v/v) BHI were used as the negative control. The culture media were collected and investigated for MMP-2 activation by gelatin zymography.

The results demonstrated that, in the negative control, fibroblasts produced and secreted pro-MMP-2 in the culture medium. Gelatin zymography showed a clear band of pro-MMP-2 at approximately 72 kDa. The experimental groups, in which cultured fibroblasts were stimulated with *E. faecalis* supernatant (ATCC 19433 and ATCC 14506), revealed three clear bands at 72 kDa, 68 kDa and 62 kDa, which correspond to the pro-MMP-2, intermediate-MMP-2 and active-MMP-2, respectively (Figure 3.1A). The *E. faecalis* supernatant of ATCC 19433 and ATCC 14506 induced MMP-2 expression. The amount of pro-MMP-2 increased and MMP-2 activation was up-regulated in a dose-dependent fashion (Figure 3.1B). This dose-dependence suggests that the response of fibroblasts in terms of MMP-2 activation depends on the concentration of secreted molecules from *E. faecalis* supernatant.

3.1.3 Regulation of MMP-2, MT1-MMP and TIMP-2 expression in cultured fibroblasts by *E. faecalis* supernatant : RT-PCR and Western blot analysis

RT-PCR was used for the analysis of MMP-2, MT1-MMP, and TIMP-2 mRNA expression after cultured fibroblasts were stimulated with various concentrations of *E. faecalis* supernatant for 48 hours (Figure 3.2 and Table 3.2). The results showed that MMP-2 and TIMP-2 expression increased with concentration, and also increased compared to that of the control group, whereas MT1-MMP expression was not altered compared to that of the control group. The increase in MMP-2 mRNA expression corresponded to the results from gelatin zymography. The

amounts of pro-MMP-2 in the experimental group were greater than those in the negative control group. These results suggest that *E. faecalis* supernatant affects the transcriptional level of MMP-2.

In the western blot analysis (Figure 3.3 and Table 3.3), the secreted TIMP-2 level in the culture media, in which cultured fibroblasts were treated with *E. faecalis* supernatant, was increased in a dose-dependent manner. The expression of MT1-MMP in lysated cell was not altered compared to the control groups. These results were similar to the results from RT-PCR.

Table 3.1 Cytotoxicity of *E. faecalis* supernatant on cultured fibroblasts

Cytotoxicity of *E. faecalis* supernatant was determined by MTT assay. Fibroblasts were stimulated with 0, 2, 10, 15, 20, 30, and 40 % (v/v) of *E. faecalis* supernatant for 24 hours. Data present the percentage of relative viable cell numbers as median from three independent experiments. Toxicity of *E. faecalis* supernatant was observed at ≥ 20% (v/v). Asterisks (*) denotes any statistically significant differences when compared with the control groups. Statistical analysis was performed using the Kruskal-Wallis H-test and post-hoc test with the Mann-Whitney U-test at $p < 0.05$.

Cultured fibroblasts were stimulated with *E. faecalis* supernatant (% v/v)	**Percentage of relative viable cell numbers (median)**	***p*-value**
Control		
in serum free medium	100.00	-
in BHI 15%	97.14	0.286
ATCC 19433 2%	104.18	0.201
ATCC 19433 10%	100.48	0.873
ATCC 19433 15%	102.45	0.394
ATCC 19433 20%	83.30	0.004*
ATCC 19433 30%	62.85	0.004*
ATCC 19433 40%	43.22	0.011*
ATCC 14506 2%	101.99	0.394
ATCC 14506 10%	98.73	0.631
ATCC 14506 15%	100.54	1.000
ATCC 14506 20%	87.44	0.004*
ATCC 14506 30%	83.72	0.004*
ATCC 14506 40%	65.52	0.011*

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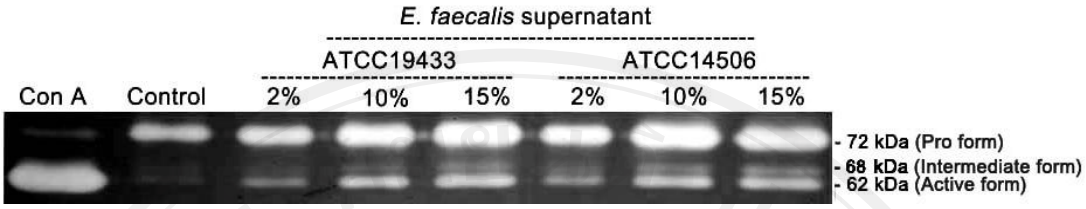

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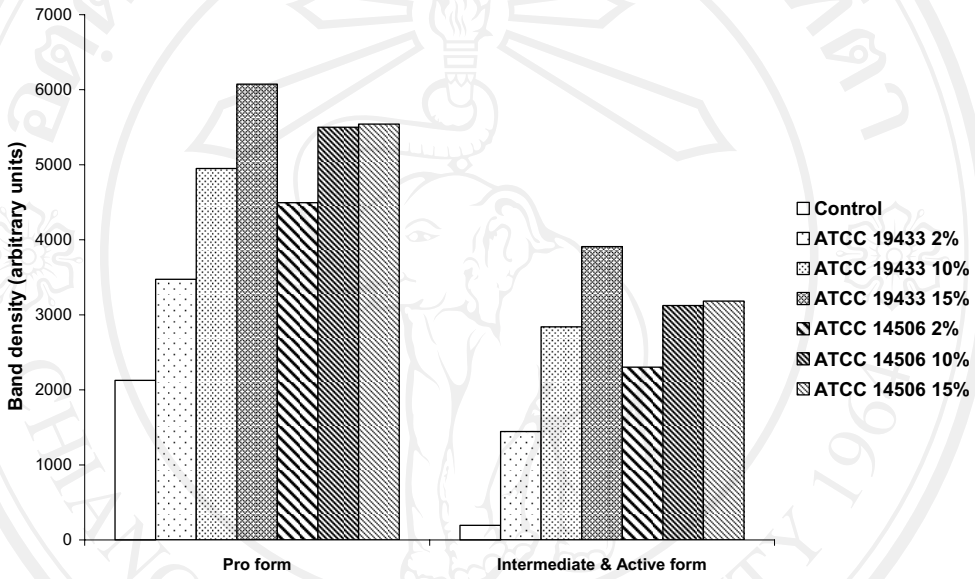

Figure 3.1 Gelatin zymography of MMP-2 activation by *E. faecalis* supernatant

(A) Fibroblasts were cultured with various concentrations of *E. faecalis* supernatant (2, 10, and 15 % (v/v)) for 48 hours. Twenty-five µg/ml of Con A was used as the positive control. Fifteen percent (v/v) of BHI was added in the culture for the negative control. The culture media were collected for gelatin zymography. The MMP-2 activation can be clearly detected when stimulated with 2, 10, and 15% (v/v) of *E. faecalis* supernatant. Positions of pro-MMP-2, intermediate-MMP-2 and active-MMP-2 (72, 68 and 62 kDa) are indicated on the right. (B) The histogram presents the amounts of pro-MMP-2 and intermediate- and active-MMP-2 forms in band density (arbitrary units).

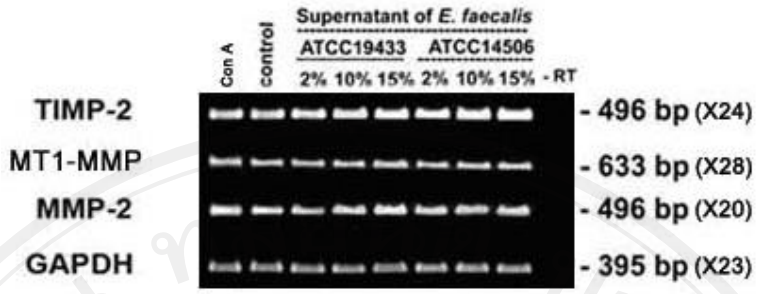

Figure 3.2 RT-PCR analysis of MMP-2, MT1-MMP and TIMP-2 expression after stimulating cultured fibroblasts with *E. faecalis* supernatant

Fibroblasts were cultured with various concentrations of *E. faecalis* supernatant (2, 10, and 15 % (v/v)) for 48 hours. Total cellular RNA was extracted, and mRNA of MMP-2, MT1-MMP and TIMP-2 was converted to cDNA by reverse transcription (RT). The cDNA was subsequently amplified by polymerase chain reaction (PCR). GAPDH was used as an internal control. –RT was a negative control where the reverse transcriptase was omitted.

Table 3.2 Relative density of MMP-2, MT1-MMP and TIMP-2 mRNA expression after stimulating cultured fibroblasts with *E. faecalis* supernatant

Data are presented in median from three independent experiments.

Cultured fibroblasts were stimulated with *E. faecalis* supernatant (% v/v)	**Relative density (median)**		
	MMP-2	**MT1-MMP**	**TIMP-2**
Control	1.00	1.00	1.00
ATCC 19433 2%	1.07	1.00	0.99
ATCC 19433 10%	1.15	1.01	1.02
ATCC 19433 15%	1.23	1.13	1.16
ATCC 14506 2%	1.12	1.10	1.10
ATCC 14506 10%	1.18	1.08	1.17
ATCC 14506 15%	1.22	1.12	1.23

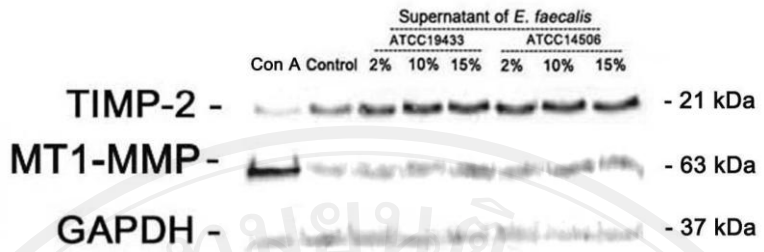

Figure 3.3 Western blot analysis of MT1-MMP and TIMP-2 after stimulating cultured fibroblasts with *E. faecalis* supernatant

Fibroblasts were cultured with various concentrations of *E. faecalis* supernatant (2, 10, and 15 % (v/v)) for 48 hours. For TIMP-2 analysis, the culture media were collected and cell debris were removed by centrifugation, and the culture media were then concentrated by a concentrator. Lysated cultured fibroblasts were used for MT1-MMP expression. The expression of GAPDH was used as an internal control. –RT was a negative control where the reverse transcriptase was omitted.

Table 3.3 Relative density of MT1-MMP and TIMP-2 protein expression after stimulating cultured fibroblasts with *E. faecalis* supernatant

Data are presented in median from three independent experiments.

Cultured fibroblasts were stimulated with *E. faecalis* supernatant (% v/v)	**Relative density (median)**	
	MT1-MMP	**TIMP-2**
Control	1.00	1.00
ATCC 19433-2%	1.15	1.34
ATCC 19433-10%	1.19	1.74
ATCC 19433-15%	1.20	1.91
ATCC 14506-2%	1.19	1.58
ATCC 14506-10%	1.21	1.79
ATCC 14506-15%	1.22	1.86

3.2 Effects of heat-killed *E. faecalis* on MMP-2 expression and activation in cultured fibroblasts from human periodontal ligament

3.2.1 Cytotoxicity of heat-killed *E. faecalis* on cultured fibroblasts : MTT assay

Cytotoxicity of heat-killed *E. faecalis* was determined by MTT assay. Fibroblasts were cultured in the presence of heat-killed *E. faecalis*, either ATCC 19433 or ATCC 14506, at various concentrations (1:10-1:10,000 ratio between number of fibroblasts and number of heat-killed *E. faecalis* bacteria) for 24 hours. Table 3.4 shows that non-toxicity of heat-killed *E. faecalis* was demonstrated at a concentration of $\leq$ 1:1,000 of both ATCC strains. No significant difference in viable cell numbers was found when cells were treated with 1:10, 1:100 or 1:1,000 ($p > 0.05$) concentrations of either ATCC 19433 or ATCC 14506 strain. However, the number of cells significantly decreased at 1:10,000 concentration of heat-killed *E. faecalis* ATCC 19433 and ATCC 14506 ($p = 0.001$, 0.000, respectively), indicating the cytotoxicity of heat-killed *E. faecalis*. Therefore, 1:10, 1:100, and 1:1,000 concentrations of heat-killed *E. faecalis* of both ATCC strains were used for the next experiments.

3.2.2 Regulation of MMP-2 expression and activation in cultured fibroblasts by heat-killed *E. faecalis* : Gelatin zymography

Fibroblasts were cultured in the presence of 1:10, 1:100 and 1:1,000 concentrations of heat-killed *E. faecalis*, either ATCC 19433 or ATCC 14506, for 48 hours. The positive control consisted of cultured fibroblasts with 25 µg/ml of Con A. The negative control consisted of non-stimulated cultured fibroblasts. The culture media were collected and MMP-2 activation was investigated by gelatin zymography.

Pro-MMP-2 (72 kDa) was detected in the negative control group and in the experimental group, whereas intermediate-MMP-2 (68 kDa) and active-MMP-2 (62 kDa) were faintly visible in the cultured fibroblasts stimulated with 1:10 and 1:100 concentrations of heat-killed *E. faecalis*. Active MMP-2 was clearly visible in the culture that contained the 1:1,000 concentration of heat-killed *E. faecalis* of both reference strains (Figure 3.4A). Pro-MMP-2, intermediate-MMP-2 and active-MMP-2 were increased corresponding to concentration of heat-killed *E. faecalis* (Figure 3.4B). These results demonstrate that heat-killed *E. faecalis* ATCC 19433 and ATCC 14506 can induce MMP-2 activation in a dose-dependent manner.

3.2.3 Regulation of MMP-2, MT1-MMP and TIMP-2 expression in cultured fibroblasts by heat-killed *E. faecalis* : RT-PCR and Western blot analysis

RT-PCR was used for the analysis of MMP-2 mRNA expression and MT1-MMP mRNA expression after cultured fibroblasts were stimulated with various concentrations of heat-killed *E. faecalis* for 48 hours. RT-PCR analysis revealed that heat-killed *E. faecalis* slightly increased mRNA expression of MMP-2 but MT1-MMP was not altered when compared to the control group (Figure 3.5 and Table 3.5). An increase in MMP-2 mRNA expression corresponded to increased MMP-2 protein in the culture media of the experimental groups as seen with gelatin zymography. The amounts of pro-MMP-2 in the experimental groups were greater than those of the negative control group after stimulation with heat-killed *E. faecalis*.

Western blot analysis (Figure 3.6 and Table 3.6) found that MT1-MMP protein level was not altered after stimulating with heat-killed *E. faecalis*, whereas secreted TIMP-2 protein level was slightly decreased in the culture media that

contained heat-killed *E. faecalis* at 1:10 and 1:100 concentrations, and was clearly decreased at 1:1,000 concentration of both reference strains.

Table 3.4 Cytotoxicity of heat-killed *E. faecalis* on cultured fibroblasts

Cytotoxicity of *E. faecalis* supernatant was determined by MTT assay. Fibroblasts were stimulated with 1:10, 1:100, 1:1,000 concentrations of heat-killed *E. faecalis* for 24 hours. Data present the percentage of relative viable cell numbers as median from three independent experiments. Cytotoxicity of heat-killed *E. faecalis* was observed at 1:10,000 concentration. The graphs marked with asterisks (*) denotes any statistically significant differences when compared with the control groups. Statistical analysis was performed using the Kruskal-Wallis H-test and the Mann-Whitney U-test at $p < 0.05$.

Cultured fibroblasts were stimulated with heat-killed *E. faecalis*	**Percentage of relative viable cell numbers (median)**	***p*-value**
Control	100.00	-
ATCC 19433-1:10	100.74	1.000
ATCC 19433-1:100	99.81	0.715
ATCC 19433-1:1000	100.74	0.715
ATCC 19433-1:10000	51.04	0.011*
ATCC 14506-1:10	95.35	0.100
ATCC 14506-1:100	95.45	0.100
ATCC 14506-1:1000	96.65	0.144
ATCC 14506-1:10000	47.99	0.011*

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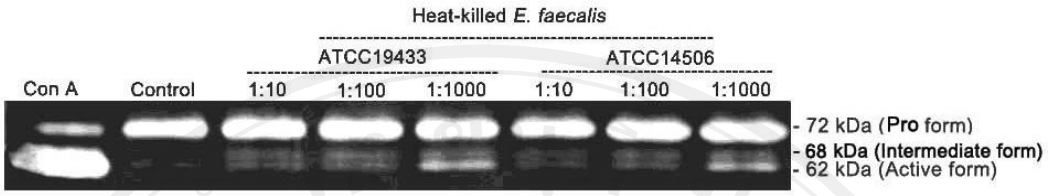

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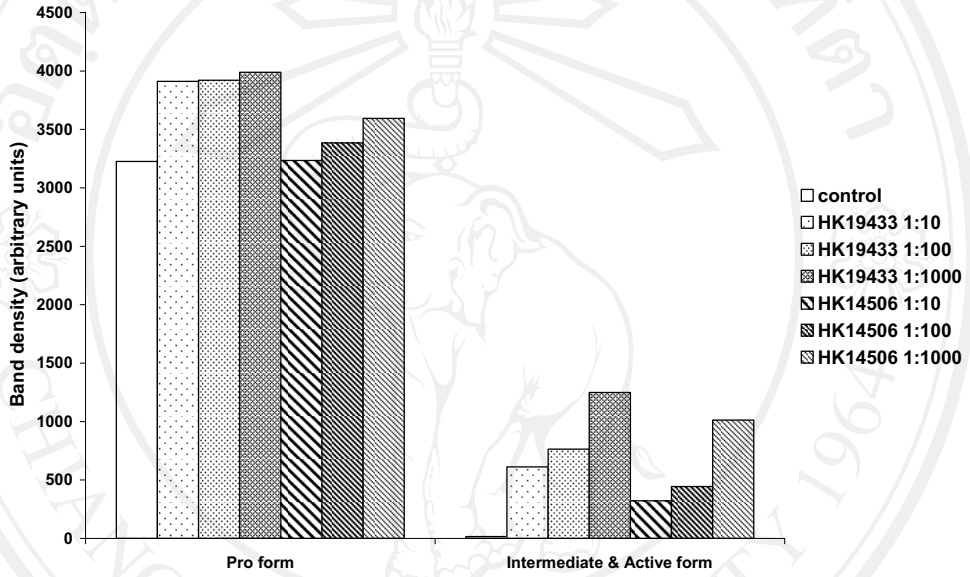

Figure 3.4 Gelatin zymography of MMP-2 activation by heat-killed *E. faecalis*

(A) Fibroblasts were cultured with various concentrations of heat-killed *E. faecalis* (1:10, 1:100, 1:1,000) for 48 hours. Twenty-five µg/ml of Con A was used as the positive control. Non-stimulated cultured fibroblasts were used for the negative control. The culture media were collected for gelatin zymography. The activation can be clearly detected when stimulated with 1:1,000 while faintly visible when stimulated with 1:10 and 1:100 of heat-killed *E. faecalis*. The positions of pro-MMP-2, intermediate-MMP-2 and active-MMP-2 (72, 68 and 62 kDa) are indicated on the right. (B) The histogram presents the amounts of pro-MMP-2 and intermediate- and active-MMP-2 forms in band density (arbitary units).

Figure 3.5 RT-PCR analysis of MMP-2 and MT1-MMP after stimulating cultured fibroblasts with heat-killed *E. faecalis*

Fibroblasts were cultured with various concentrations of heat-killed *E. faecalis* (1:10, 1:100 and 1:1,000) for 48 hours. Total cellular RNA was extracted, and mRNA of MMP-2 and MT1-MMP was converted to cDNA by reverse transcription (RT). The cDNA was subsequently amplified by polymerase chain reaction (PCR). The expression of GAPDH was used as an internal control. –RT was a negative control where the reverse transcriptase was omitted.

Table 3.5 Relative density of MMP-2 and MT1-MMP mRNA expression after stimulating cultured fibroblasts with heat-killed *E. faecalis*

Data are presented in median from three independent experiments.

Cultured fibroblasts were stimulated with heat-killed *E. faecalis*	**Relative density (median)**	
	MMP-2	**MT1-MMP**
Control	1.00	1.00
ATCC 19433 1:10	1.11	1.07
ATCC 19433 1:100	1.15	1.07
ATCC 19433 1:1000	1.20	1.07
ATCC 14506 1:10	1.15	0.98
ATCC 14506 1:100	1.12	1.01
ATCC 14506 1:1000	1.09	1.03

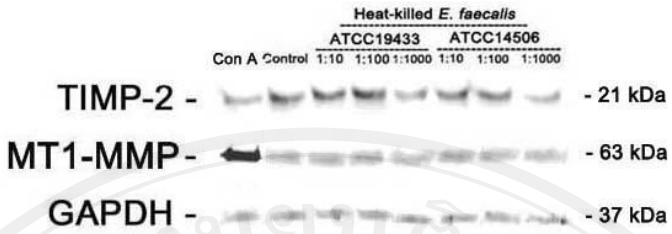

Figure 3.6 Western blot analysis of MT1-MMP and TIMP-2 after stimulating cultured fibroblasts with heat-killed *E. faecalis*

Fibroblasts were cultured with various concentrations of heat-killed *E. faecalis* (1:10, 1:100 and 1:1,000) for 48 hours. For TIMP-2 analysis, the culture media were collected, and cell debris was removed by centrifugation, and the media were then concentrated by a concentrator. Lysated cultured fibroblasts were used for MT1-MMP expression. The expression of GAPDH was used as an internal control.

Table 3.6 Relative density of MT1-MMP and TIMP-2 protein expression after stimulating fibroblasts with heat-killed *E. faecalis*

Data are presented in median from three independent experiments.

Cultured fibroblasts were stimulated with heat-killed *E. faecalis*	**Relative density (median)**	
	MT1-MMP	**TIMP-2**
Control	1.00	1.00
ATCC 19433-1:10	1.01	1.01
ATCC 19433-1:100	1.06	0.96
ATCC 19433-1:1000	1.08	0.84
ATCC 14506-1:10	1.03	0.94
ATCC 14506-1:100	1.07	0.91
ATCC 14506-1:1000	0.99	0.65