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Determination of antibacterial activities of lauric acid, monolaurin and the combination against MRSA, Streptococcus suis, and Clostridum perfringens.

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Project numer: 4417797730

Introduction

Following the results on the antibacterial effect of lauric acid and monolaurate (WLR project 4417797070 described in CVI report number: 11 / CVI0167) has been decided in consultation with the client to start a next phase of the research. In this second phase the antibacterial action of a combination of lauric acid (LZ) and monolaurate (ML) is investigated (ratio: 80% LZ and 20% ML) compared to the MRSA, S. suis and C. perfringens chosen in phase 1. isolates.

Material and Method

Test substances

- Lauric acid, batch number: 2011018, content C12: > 95%

- Monolaurate, batch number: M31027455, content of monoglycerides: 99.8%

Media

- Mueller Hinton broth (MHB), pH 6.5 (buffered with a 0.05M phosphate buffer)

- Physiological saline solution (0.85%)

- HIS (Heart Infusion) agar plates + 5% sheep blood

Bacterium isolates

- Methicillin resistant Staphylococcus aureus (MRSA), number 12.03 isolated from pig in 2010.

- Methicillin resistant Staphylococcus aureus (MRSA), number 14.17 isolated from pig in 2010.

- Streptococcus suis, serotype 2, reference strain 5211

- Streptococcus suis, serotype 9, reference strain 5218

- Clostridium perfringens type A, number 10007252-1 isolated from alpaca in 2010

- Clostridium perfringens type C, number 10005148-1 isolated from pig in 2010

Test Method

Making the stock solutions and dilution series

From the pure individual substances lauric acid (LZ) and monolaurate (ML) a fresh stock solution was made with a combined total concentration of 51.2 mg / ml in pure ethanol (> 99.5%) consisting of 80% LZ (40.96 mg / ml) + 20% ML (10.24 mg / ml). Appendix 2 (table 7) shows the concentrations of the individual substances. Based on a high purity of the substances tested, no correction was made for impurities. The freshly made stock solutions were diluted 100 x in Mueller Hinton broth (pH 6.5 ± 0.1) to the highest concentration of 512 mg / L to be tested. Subsequently, a 2-fold dilution series was made per substance in tubes with 3 ml buffered Mueller Hinton broth (MHB) to a concentration of 4 mg / l, after which the following 8 concentrations were tested: 512, 256, 128, 64, 32, 16, 8, and 4 mg / L.

The stock solutions of the LZ / ML combination in ethanol were completely clear.

After the preparation of the dilution series, turbidity was seen in MHB at the three highest concentrations (512, 256 and 128 mg / L) both in the LZ / ML combination. From 64 mg / L no turbidity was observed due to the substances.

Inoculation

From a fresh clean culture on a HIS plate, a suspension was made of each bacterium in a physiological saline solution (0.85% NaCl) with a standard turbidity (0.5 McFarland) using a turbid meter. This suspension corresponds to a concentration of about $1x10^8$ CFU / ml. Subsequently, the 3 ml MHB tubes were inoculated with 15 µl of the 0.5 McFarland suspension (initial concentration approximately 5.10^5 CFU / ml).

Growth controls

During each test a growth control blank group was included by inoculating MHB tube per bacterium isolate without the test substance. In addition, an additional blank growth control was included as MHB + 1% ethanol per bacterium isolate. This concentration of ethanol corresponds to the concentration of ethanol as the solvent in the highest concentration of the substances to be tested (512 mg / L). Finally, a non-inoculated MHB tube was included as a negative control during each test.

pH controls

The buffering capacity of the Mueller Hinton broth was determined by measuring the pH value of a dilution series of the test substances tested after incubation.

Visual reading of the MIC

After 20 - 24 hours of incubation at 35°C (aerobic at 35°C for S. suis and MRSA and anaerobic at 35°C for C. perfringens) the tubes were evaluated for visible bacterial growth compared to negative control tubes. The lowest concentration of the test

substance without visible bacterial growth was noted as the Minimum Inhibitory Concentration (MIC).

Insert and read germ counts

After incubation, each MHB tube was diluted 10-fold in physiological saline (f.z.; fysiologische zoutoplossing) to 10^{-6} by mixing 200 µl each time with 1.8 ml f.z. 100 µl of each dilution was placed on a HIS plate and peeled out. After incubation (18-24 hours at 35°C) the colonies were counted and then the bacteria count calculated on the basis of the number of colonies on the plate with between 30 and 300 colonies. Based on these bacteria count, a derived MIC value was determined by recording the lowest concentration at which the bacteria count was equal to or lower than the concentration of the starting bacteria count.

Results

To compare the MICs of the individual substances with the combination of lauric acid and monolaurate, the MICs of the individual substances as determined in phase 1 are shown again in Tables 1 and 2.

Table 1. MICs in mg / L of monolaurate (ML) and lauric acid (LZ) and the combination of the two substances (80% LZ / 20% ML) based on visual reading

	ML (1st)	ML (2nd)	LZ (1st)	LZ (2nd)	LZ/ML (1^{st})	LZ/ML(2nd)
Isolates	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
MRSA 12.03	32	32	64	128	64	32
MRSA 14.17	32	32	128	128	64	64
S. suis 5211	16	16	16	16	8	8
S. suis 5218	16	16	32	32	8	8
C. perfringens 5148-1	16	32	64	32	32	32
C. perfringens 7252-1	16	16	64	64	32	32

Table 2. MICs of monolaurate (ML) and lauric acid (bacteria count

	ML (1st)	ML (2nd)	LZ (1st)	LZ (2nd)	LZ/ML(1^{st})	LZ/ML(2nd)
Isolates	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
MRSA 12.03	32	32	128	128	64	32
MRSA 14.17	32	32	128	128	64	64
S. suis 5211	16	16	32	32	16	16
S. suis 5218	16	16	32	32	16	8
C. perfringens 5148-1	16	32	64	32	32	32
C. perfringens 7252-1	16	16	64	64	32	32

Table 3. Bacteria count of MRSA 12.03 and 14.17 after 24 hours in lauric acid / monolaurate (LZ / ML)

LZ/ML (mg/L)	MRSA 12.03-A	MRSA 12.03-B	MRSA 14.17-A	MRSA 14.17-B
0	5.20E+08	4.10E+08	5.00E+08	8.20E+08
4	3.90E+08	2.10E+08	5.40E+08	5.50E+08
8	3.50E+08	6.60E+08	5.90E+08	6.00E+08
16	2.40E+08	2.50E+08	5.00E+08	4.20E+08
32	2.90E+06	2.10E+05	1.30E+07	2.10E+07
64	2.90E+02	2.00E+01	4.00E+01	1.00E+01
128	1.00E+01	1.00E+01	1.00E+01	1.00E+01
256	1.00E+01	1.00E+01	1.00E+01	1.00E+01
512	1.00E+01	1.00E+01	1.00E+01	1.00E+01
Starting count	3.1E+05	5.3E+05	4.10E+05	4.30E+05

The MICs derived from the bacteria count are highlighted in yellow.

Figure 1. Bacteria count of MRSA after 24 hours in lauric acid / monolaurate (LZ / ML)

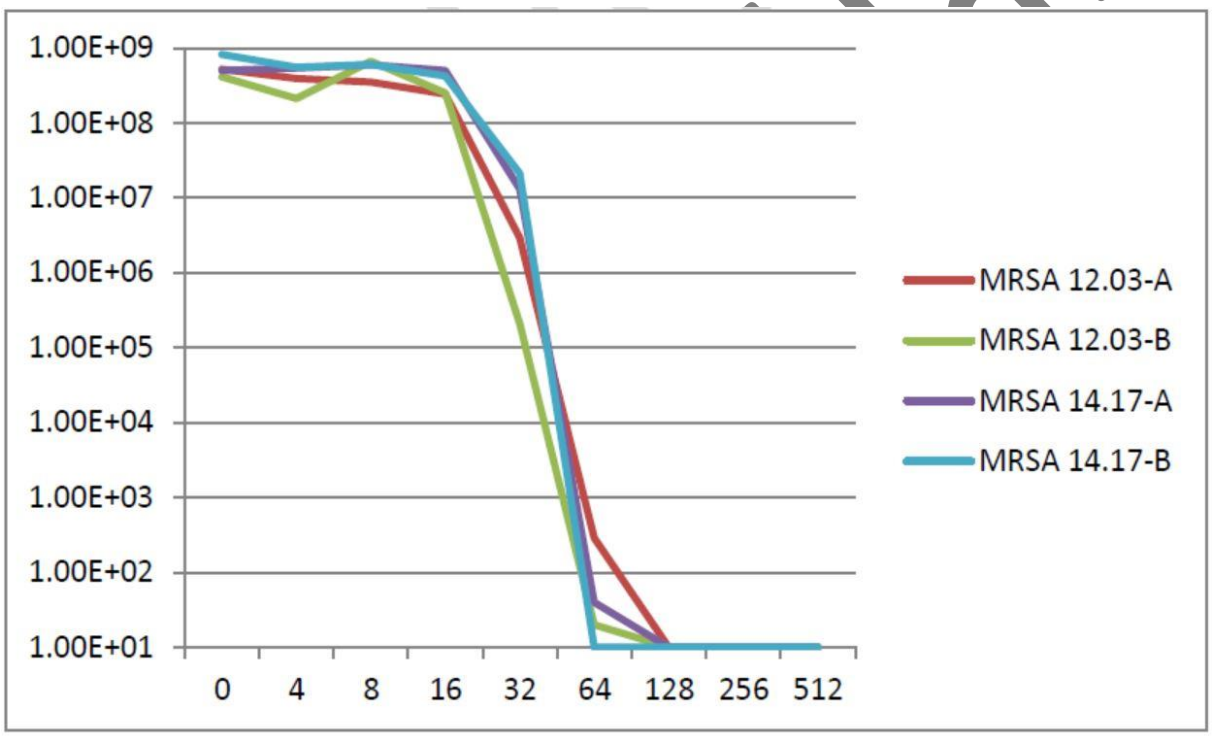


Table 4. Bacteria count of S. suis 5211 and 5218 after 24 hours in lauric acid / monolaurate (LZ / ML)

LZ/ML (mg/L)	S.suis 5211-A	S.suis 5211-B	S.suis 5218-A	S.suis 5218-B
0	1.80E+08	2.5E+08	1.00E+08	9.00E+07
4	4.00E+06	1.10E+07	1.00E+08	7.70E+06
8	7.80E+06	1.70E+07	7.50E+05	2.50E+05
16	4.20E+04	7.70E+04	2.20E+05	6.00E+04
32	1.00E+01	1.00E+01	4.50E+03	7.80E+02
64	1.00E+01	1.00E+01	1.00E+01	1.00E+01
128	1.00E+01	1.00E+01	1.00E+01	1.00E+01
256	1.00E+01	1.00E+01	1.00E+01	1.00E+01
512	1.00E+01	1.00E+01	1.00E+01	1.00E+01
Starting count	1.30E+06	1.10E+06	6.50E+05	5.40E+05

The MICs derived from the bacteria count are highlighted in yellow.

Figure 2. Bacteria count of S. suis after 24 hours in lauric acid / monolaurate (LZ / ML)

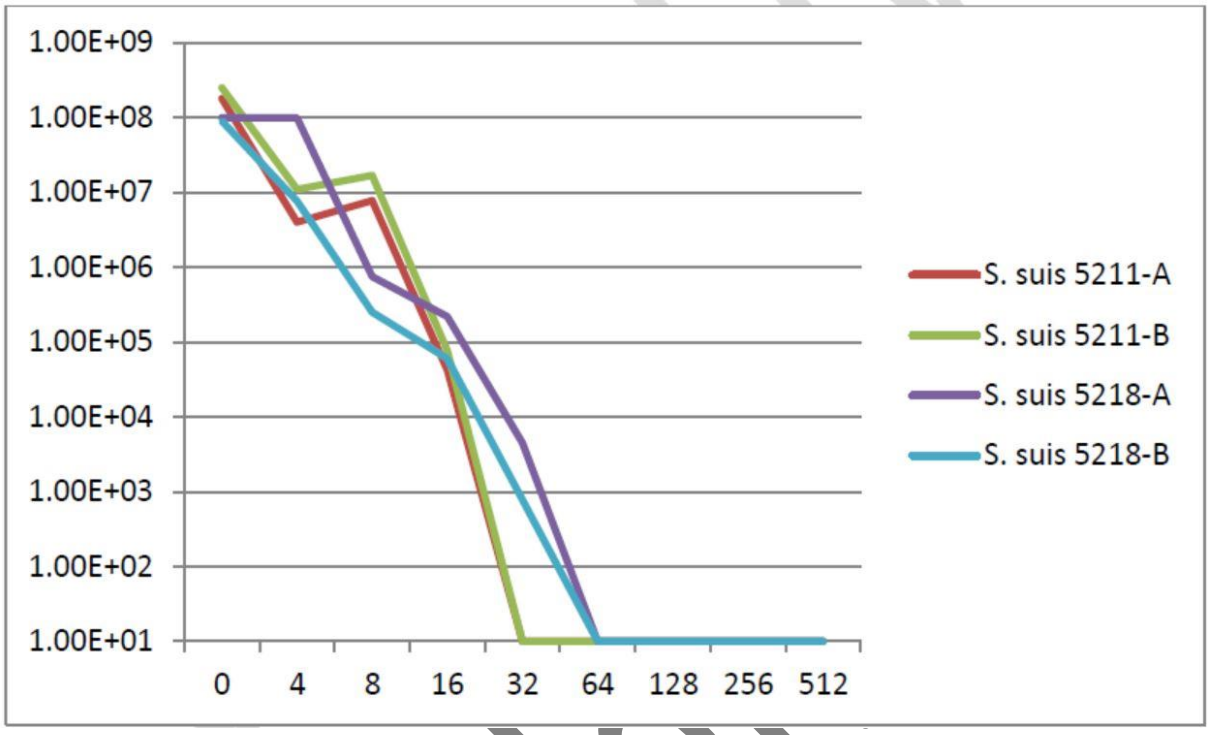
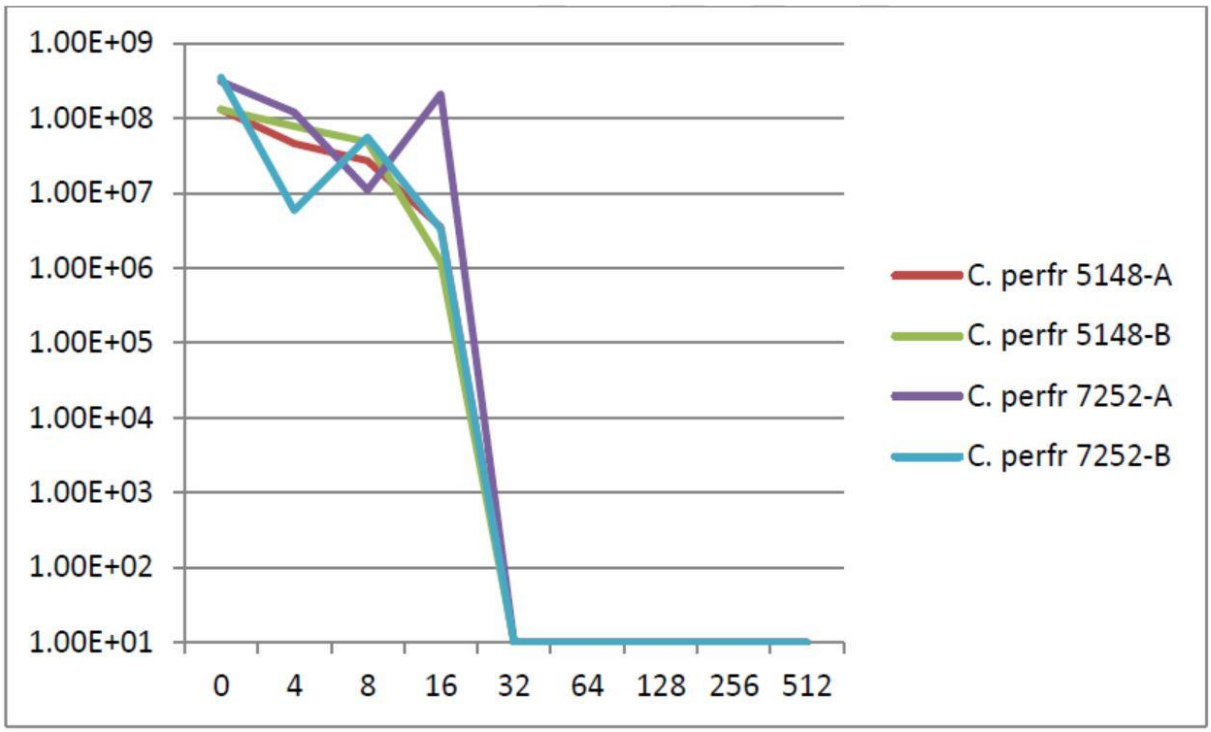


Table 5. Bacteria count of C. perfringens 10005148-1 and 10007252-1 after 24 hours in lauric acid / monolaurate (LZ / ML)

LZ/ML (mg/L)	C. perfr 5148-A	C. perfr 5148-B	C. perfr 7252-A	C. perfr 7252-B
0	1.30E+08	1.30E+08	3.10E+08	3.50E+08
4	4.60E+07	7.80E+07	1.20E+08	5.90E+06
8	2.70E+07	4.80E+07	1.10E+07	5.60E+07
16	3.50E+06	1.20E+06	2.10E+08	3.30E+06
32	1.00E+01	1.00E+01	1.00E+01	1.00E+01
64	1.00E+01	1.00E+01	1.00E+01	1.00E+01
128	1.00E+01	1.00E+01	1.00E+01	1.00E+01
256	1.00E+01	1.00E+01	1.00E+01	1.00E+01
512	1.00E+01	1.00E+01	1.00E+01	1.00E+01
Starting count	5.70E+04	1.20E+05	1.70E+05	2.00E+05

The MICs derived from the bacteria count are highlighted in yellow.

Figure 3. Bacteria count of C. perfringens after 24 hours in lauric acid / monolaurate (LZ / ML)



Discussion and conclusion

Control of the bacteria counts

The variation in the bacteria counts of the growth controls of the MRSA isolates was relatively small. For MRSA 12.03, the bacteria count ranged between 4.10^8 - 5.10^8 cfu / ml and for MRSA 14.17 between 5.10^8 - 8.10^8 cfu / ml. No growth inhibition was found in MHB + 1% ethanol.

With S. suis larger differences were seen in the bacteria counts of the growth controls. For S. suis 5211 the bacteria count varied between 1.10^8 - 2.10^8 cfu / ml, but for S. suis 5218 these values were lower and the variation was greater: 6.10^7 cfu / ml and 1.10^8 cfu / ml. For both S. suis isolates growth inhibition was determined in MHB with 1% ethanol to a maximum of two log-steps growth reduction (in S. suis 5211). For this reason, the growth of both S. suis isolates in different ethanol concentrations was tested in MHB. This showed that no growth inhibition of S. suis occurred at 0.5% ethanol. The MICs of the S. suis isolates for the different substances ranged from 8 - 64 mg / L. This corresponds to an ethanol percentage of 0.03 - 0.125%, with which the influence of the ethanol on the MIC provisions may be negligible.

In C. perfringens the mutual difference and the variation between the bacteria counts was relatively small. The bacteria count of both isolate 5148-1 and isolate 7252-1 varied between 1.10^8 - 3.10^8 cfu. For C. perfringens no growth inhibition was observed in MHB + 1% ethanol.

Turbidity of the tubes through the substances tested

Only the highest three concentrations of the investigated substances (512, 256 and 128 mg / L) showed turbidity in MHB; yet, visual assessment of bacterial growth was possible to determine in most cases after incubation. However, the determination of the bacteria counts had certainly provided more accuracy, because the turbidity in the tubes with both S. suis isolates and with C. perfringens 5148-1 was difficult to assess. In spite of this, the differences in the MIC values between the visual reading and the MICs on the basis of the bacteria counts were small with a maximum variation of 1 concentration step. In view of the difficulties with the visual reading, the MICs derived from the germ count were chosen for the further evaluation of the results.

Discussion results

Combination lauric acid / monolaurate

For the combination of 80% lauric acid / 20% monolaurate (LZ / ML), an antibacterial effect has been observed with respect to all three bacterial species studied. The measured MIC values were between 8 - 64 mg / L. The measured antibacterial activity of the LZ / ML combination was found to be stronger than lauric acid (LZ) and in most cases less strong than monolaurate (ML). For the LZ / ML combination, MIC values were measured in relation to the MRSA isolates, which varied between 32- 64 mg / ml. These values were between the MIC values found in phase 1 for lauric acid (128 mg / L) and monolaurate (32 mg / L). The lowest MIC values for the LZ / ML combination (8-16 mg / L) were measured with respect to S. suis. In S. suis, the MIC values of the combination corresponded to the values previously measured for monolaurate (MIC: 16 mg / L). In C. perfringens the MIC values of the LZ / ML combination (32 mg / L) were between the previously measured MIC values for lauric acid (32-64 mg / L) and monolaurate (16- 32 mg / L). In only two cases a difference of one concentration step was found between the duplicates (in MRSA 12.03 and S. suis 5218). This shows that the procedures have been made reproducible. After all, a variation of one step around the average falls within the normal variation of a MIC determination.

pH control

Table 6 in appendix 1 shows that the pH influence of the investigated substances was negligible. The highest pH drop (- 0.05) was measured at the highest concentration of LZ / ML. The results show that a possible pH change as a result of the added substances did not influence the outcome of the determinations.

Appedix 1

Table 6 pH control of Mueller Hinton bouillon (MHB)

Substances	concentration	pH
Blank	-	6.41
Blank +1% ethanol	-	6.42
LZ/ML	4	6.39
LZ/ML	8	6.40
LZ/ML	16	6.40
LZ/ML	32	6.40
LZ/ML	64	6.39
LZ/ML	128	6.39
LZ/ML	256	6.37
LZ/ML	512	6.36

Appendix 2

Table 7. Individual concentrations of lauric acid and monolaurate in the tested concentration range of a mixture with 80% lauric acid and 20% monolaurate (LZ / ML)

Total concentration LZ/ML (mg/ L)	Lauric acid (mg/L)	GML (mg/L)
512	409.6	102.4
256	204.8	51.2
128	102.4	25.6
64	51.2	12.8
32	25.6	6.4
16	12.8	3.2
8	6.4	1.6
4	3.2	0.8