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► **To cite this version:**

Stéphane Corvec, Eva Seiler, Lei Wang, Mercedes Gonzalez Moreno, Andrej Trampuz. Characterization of medical relevant anaerobic microorganisms by isothermal microcalorimetry. *Anaerobe*, 2020, 66, pp.102282 -. 10.1016/j.anaerobe.2020.102282 . hal-03492861

HAL Id: hal-03492861

<https://hal.science/hal-03492861v1>

Submitted on 17 Oct 2022

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1 **Research article Anaerobe V2**

2

3 **Characterization of medical relevant anaerobic microorganisms**

4 **by isothermal microcalorimetry**

5

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15

16 **Running title:** Detection of anaerobes by calorimetry

17 **Keywords:** anaerobes, detection, calorimetry

18 **Word count (Introduction through Discussion):** 2592

19

20 Potential conflicts of interest: None reported.

21

22

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1 Financial support: The study was supported by an unrestricted educational grant from the PRO-
2 IMPLANT Foundation (<https://www.pro-implant.org>), a non-profit organization supporting
3 research, education, global networking and care of patients with bone, joint or implant-associated
4 infection.

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14

1 **ABSTRACT 227words**

2 Detection of anaerobe bacteria by culture methods requires appropriate media, special growth
3 conditions, additional detection techniques and it typically takes several days. Therefore,
4 anaerobes are often missed in patient specimens under routine culture conditions.
5 Microcalorimetry may provide a simple and accurate real-time method for faster and better
6 detection of anaerobes. An isothermal calorimeter was used for calorimetric experiments, which
7 detect minimal changes of temperature over time. In order to find optimal growth conditions,
8 seven reference or clinical strains of medical relevant anaerobe bacteria were tested under
9 different circumstances. First, the strains were tested with different growth media. After
10 determining the optimal medium for each strain, the gas phase was modified by adding 3 mL or 4
11 mL medium, to evaluate growth under conditions with less oxygen. Cooked Meat Medium was
12 best supporting growth of the tested strains, including *Cutibacterium acnes*, *Fusobacterium*
13 *nucleatum*, *Finnegoldia magna*, *Parvimonas micra*, *Bacteroides fragilis* and *Actinomyces*
14 *odontolyticus*, followed by thioglycolate. The best medium to detect *Clostridioides difficile* was
15 H-Medium. All tested strains showed better growth in 4 mL medium than in 3 mL. The detection
16 time ranged between 10 to 72 hours. Our results demonstrated that the sensitivity and the
17 detection time of anaerobe bacteria can be improved by isothermal calorimetry with optimization
18 of growth conditions. Therefore, calorimetric detection, a practical, quick and easy-to-do method,
19 has the potential to replace current microbiological methods.

20

1 **1. Introduction**

2 Anaerobe bacteria are part of the normal human flora in the oral cavity, upper respiratory tract,
3 lower intestinal tract, genito-urinary tract and on the skin [1]. Anaerobes are involved in several
4 infections, predominantly causing polymicrobial infections together with aerobic bacteria [2,3]. It
5 is important to identify anaerobes, as they often require other or additional antibiotics [4].

6 In routine microbiology, anaerobes are detected either by anaerobic cultures, by molecular testing
7 or by identifying their specific toxin, e.g. from feces for *Clostridioides difficile* [5,6]. Cultures
8 may require a CO₂-enriched atmosphere to grow properly, and the detection process and
9 microbial identification can take several days (e.g. *Actinomyces* spp.). Therefore, faster and
10 improved detection of anaerobes may be of advantage in the routine microbiological diagnosis. In
11 addition, knowledge of optimal growth conditions, growth media and growth characteristics may
12 further improve the clinical practice [5].

13 Microcalorimetry was described as a new diagnostic method allowing rapid and accurate
14 detection of microorganisms by measuring their heat production as a result of their metabolism
15 [7]. Microcalorimetry was also reported as a promising tool for the diagnosis of septic arthritis
16 from synovial fluid [8] or detection of contaminated platelet products [9]. The output of the
17 calorimeter is the rate of change of heat as a function of time in the units of power (Joule/s =
18 Watt), which is referred to as a power-time curve. Despite of the low heat output of bacterial
19 metabolism, the capacity of bacteria to replicate in culture medium leads to an exponential
20 increase of cell numbers in the ampoules, which leads to detectable sums of heat even from low
21 inocula - from 10 to 100 colony-forming units (CFU) per ampoule - within hours [10–12].

22 More than 75% of anaerobes isolated from selected clinical specimens belong to Gram-positive
23 anaerobic cocci, non-spore-forming rods, *Prevotella* spp., *Porphyromonas* spp., *Bacteroides*
24 *fragilis* group and *Fusobacterium* spp. [13]. The aim of this study was to determine the optimal

1 growth media and conditions for the highest sensitivity and shortest detection time of medically
2 important anaerobe bacteria by using microcalorimetry. The microcalorimetry data were
3 compared with the results of conventional cultures. From this *in vitro* experimental work, we
4 aimed to determine the optimal conditions for diagnosing infections caused by the tested
5 microorganisms.

6 The following representative anaerobes were investigated in this study: *Cutibacterium acnes*,
7 *Actinomyces odontolyticus*, *C. difficile*, *Parvimonas micra*, *Fingoldia magna*, *Bacteroides*
8 *fragilis*, and *Fusobacterium nucleatum*. *C. acnes* is a Gram-positive non-spore-forming rod, that
9 causes acne and endogenous infections like endocarditis or device-related infections [14–16]. *A.*
10 *odontolyticus* is a Gram-positive non-spore-forming rod causing actinomycosis, an acute or
11 chronic disease with suppurative lesions, abscesses, and draining sinus tracts [17,18]. *C. difficile*
12 is a Gram-positive spore-forming rod, causing antimicrobial-associated diarrhea, colitis or
13 pseudo-membranous colitis, associated with significant morbidity and mortality particularly in
14 the elderly [19]. *P. micra* and *F. magna* are associated with infections of the oral cavity,
15 respiratory tract, genito-urinary tract, central nervous system, superficial and soft-tissue, intra-
16 abdominal sites, cardiovascular system or bacteraemia. *F. magna* is also recovered from bone and
17 joint, soft-tissue, foot ulcer and abdominal infections [20,21]. *B. fragilis* and *F. nucleatum* are
18 Gram-negative rods. *B. fragilis* is causing peritonitis, intra-abdominal abscesses and liver-
19 abscesses, whereas *F. nucleatum* causes oro-facial, lower respiratory tract, abdomen and angina
20 Paul-Vincent [22,23].

21

22 **2. Material and methods**

23 **2.1. Preparation of the bacteria for microcalorimetry**

1 The tested strains were either clinical isolates obtained from the clinical microbiology laboratory
2 at our institution or reference laboratory strains as follows: *C. acnes* (isolate T6030), *F.*
3 *nucleatum* (isolate T6080.1), *F. magna* (isolate T5945), *P. micra* (strain ATCC 33270), *B.*
4 *fragilis* (strain ATCC 25285), *A. odontolyticus* (strain ATCC 17929), *C. difficile* (isolate 9689).
5 First, colonies from a fresh culture on Schaedler agar (bioMérieux, Marcy-l'Etoile, France) was
6 suspended in 2 mL 0.85% NaCl ampoules to give a turbidity of 0.5 McFarland (McF). From this
7 0.5 McF suspension, serial 10-fold dilutions in sterile saline were prepared to achieve a dilution
8 of 1:1000, except for *C. difficile* dilution of 1:10. Sterile 4 mL glass ampoules were filled with
9 the different test media and 50 µL of the inoculum. The ampoules were sealed with sterile
10 rubber-aluminum lids. A hook was attached on each lid to introduce the ampoule into the
11 instrument and the calorimetric measurement was started. Additionally, equivalent dilutions of
12 the inoculum were plated on Schaedler agar and incubated at 37°C during two to three days to
13 determine the inoculum by counting the number of colonies. In general, a turbidity of 0.5 McF
14 represents approximately 1.5×10^8 CFU/mL, but this value varies, mainly due to different cell
15 sizes [24].

16 **2.2. Comparing four different media**

17 Trypticase Soy Broth supplied with hemin and vitamin K (TSB-F, bioMérieux) is a standard
18 liquid culture medium, usually recommended as universal growth medium for all
19 microorganisms. This standard medium was compared with Cooked Meat Medium (CMM,
20 BBL™ Cooked Meat Medium with Glucose, Hemin and Vitamin K, Becton Dickinson, Eysins
21 Switzerland), Thioglycolate (BBL™ Enriched Thioglycolate Medium with Vitamin K and
22 Hemin, Becton Dickinson), and H-Medium (Heipha Eppelheim, Germany), three media
23 recommended for anaerobic culture. Before use, aliquots of these media were reduced by

1 exposure to an anaerobic environment or by cooking for 10 minutes. For practical reasons, the
2 preparation of the ampoules took place in an aerobic environment. The ampoules were filled with
3 3 mL of one medium plus 50 μ L of the dilution containing the bacteria.

4 The bacterial concentration in the dilutions was determined by counting the number of CFU after
5 plating 50 μ L aliquots of two dilutions on Columbia blood agar plates containing 5% sheep blood
6 (Becton Dickinson) and incubating in an anaerobic hood at 37°C for two or three days. For all
7 species, the dilutions 1:10⁴ and 1:10⁵ were plated except for *C. difficile* the dilutions 1:10 and
8 1:100 were used. Results were expressed as mean \pm standard deviation (SD).

9 **2.3. Reduced gas phase**

10 As oxygen is toxic for anaerobic bacteria, the oxygen inside the ampoule was reduced by filling it
11 with 4 mL medium instead of 3 mL, thereby reducing the gas-phase inside to a minimum. This
12 time, only the optimal medium for each strain – as formerly tested – was used. Thus for every
13 strain, there was one ampoule with 3 mL and one ampoule with 4 mL of the optimal medium plus
14 50 μ L of the dilution containing the bacteria.

15 **2.4. Calorimetric equipment and measurements**

16 A 48-channel batch calorimeter (Thermal Activity Monitor, Model 3102 TAM III, TA
17 Instruments, New Castle, DE, USA) was used to measure the heat flow at 37°C controlled at \pm
18 0.0001°C and a sensitivity of \pm 0.2 μ W. Any heat generated or absorbed by the sample as a
19 consequence of any chemical or physical process is measured continuously as a function of time.
20 Each microcalorimeter is capable of accommodating a 4 mL glass ampoule in its measurement
21 chamber above a thermally inert reference in the reference chamber. Ampoules were first
22 lowered into a thermal equilibration position for 15 minutes before being lowered into the
23 measurement position. However, even after 15 minutes in the equilibration position, the

1 measurement of heat flow is temporarily disturbed by introducing an ampoule into the
2 measurement position, due to the heat capacity of the ampoule and its contents. Typically,
3 another 45 minutes must elapse before the signal from a specimen which is not currently
4 producing or consuming heat returns to a value close to zero μW of heat flow. Heat flow rate data
5 are then recorded continuously until the experimenter chooses to end an evaluation. In the study
6 described here, heat flow from ampoules was recorded for up to several days. After the
7 measuring phase the ampoules were removed from the microcalorimeter and the content of each
8 ampoule was checked for the presence of microorganisms. Sterility of the negative controls was a
9 basic requirement for the validity of an experiment.

10 Time to detection (TTD) was defined as the time between the insertion of an ampoule into the
11 calorimeter and the increase of the power-time curve above a certain threshold value. We
12 determined this value at 10 μW above the lowest point of the curve immediately before the
13 increase leading to the peak. The detection limit was set at 20 μW in order to distinguish start of
14 microbial growth from background noise. Sterile solutions never give rise to an exponential
15 increase in the heat flow, unless replicating cells are present. Heat flow peak is defined as the
16 highest value on the Y-axis (heat-flow) of the heat flow-time curve. Total heat (in Joules [J]) was
17 defined as heat generated until a time point and was determined by integration of the area below
18 the heat flow-time curve. After measurement was completed, the content of each calorimetry
19 ampoule was assessed for turbidity. Experiments were performed in triplicates on different days
20 with different batches and bacterial inocula.

21

22 **3. Results**

23 **3.1. Comparing performances of four different media**

1 The aim of this assay was to determine the medium leading to the fastest and best growth of the
2 tested species and to check if there is one universal medium, suited for the detection of most, if
3 not all common pathogenic anaerobic bacteria. The tested media were TSB-F and CMM,
4 thioglycolate and H-Medium, media that are routinely recommended and used for culture of
5 anaerobes.

6 Table 1 shows that TSB-F was not suited to detect most of the pathogens using the 10 μ W-
7 detection limit. Using the other three media all the tested species could be detected. CMM
8 resulted in the highest mean heat flow peak in five out of seven microorganisms especially *C.*
9 *acnes* (Fig. 1a) and the TTD was the shortest for three out of seven microorganisms ranging
10 between 22 to 72 hours. Thioglycolate led to the fastest TTD for *C. acnes* and *F. nucleatum*
11 though the highest mean heat flow peak it achieved only in *F. nucleatum*. The best medium to
12 detect *C. difficile* was H-Medium (shortest TTD and highest peak, Fig.1b) and it was faster than
13 the other media to detect *B. fragilis*, with a detection time of 19.6 ± 0.8 hours. In summary, CMM
14 obtained the best coverage to detect the tested anaerobic microorganisms looking at both
15 parameters TTD and heat flow peak.

16

17 **3.2. Impact of reduced gas phase**

18 With the best medium for each species determined in the previous experiment, the detection
19 parameters were compared 4 mL *versus* 3 mL medium. For *C. acnes*, only the CMM medium
20 was tested, although there was a shorter TTD with thioglycolate but with a much lower heat.

21 With *B. fragilis* H-Medium was tested instead of CMM, because of the markedly shorter TTD.

22 Of seven tested organisms, four showed shorter TTD with using the larger volume of media,
23 whereas two species showed no significant change. For most (six of seven tested organisms) the

1 microcalorimetric peak was higher with larger volume of media, involving a minimized gas
2 phase (Table 2 and Fig.2).

3

4

5

6 **4. Discussion**

7 In this study, we investigated the potential of microcalorimetry to detect anaerobe bacteria with
8 better sensitivity and speed. An isothermal calorimeter was used for calorimetric experiments,
9 which detect minimal changes of temperature over time. To achieve this goal, different growth
10 media were artificially contaminated with a selection of seven different representative anaerobe
11 bacteria strains. Culture conditions were varied according to the specific requirements of the
12 microorganisms. The calorimetric results were compared with conventional microbiological tests.
13 As previously shown in the literature [24,25], we found that CMM was the optimal supporting
14 growth medium for the tested anaerobes, including *C. acnes*, *F. nucleatum*, *F. magna*, *P. micra*,
15 *B. fragilis* and *A. odontolyticus*, followed by thioglycolate. The best medium to detect *C. difficile*
16 was H-Medium [26].

17 In order to reduce the amount of oxygen in the samples, we reduced the media before use by
18 either incubating them in anaerobic atmosphere or cooking for 10 min. Additionally, the
19 ampoules were filled by using a volume of 4 mL instead of 3 mL, to maximally reduce the gas
20 phase in the ampoule. To further decrease the oxygen level in the ampoules, all preparation steps
21 for the filling of microcalorimetry ampoules could be performed under anaerobic conditions,
22 using a special hood, or the gas phase in the ampoules could be exchanged by nitrogen. These
23 measures could further improve the detection time, especially for strict anaerobes [27].

24 In this study, we optimized the conditions for different clinical relevant anaerobe microorganisms

1 to better detect them by growth-related heat production. Once there is a microcalorimetry setting
2 with optimal conditions, (i.e. optimal medium and optimal volume of the medium), microbial
3 detection can be performed with high sensitivity and specificity. Moreover, the heat-flow curve
4 of each microorganism is distinguishable from the others (data not shown), which additionally
5 might be further explored for accurate identification of the group or even species of
6 microorganism in patient samples with suspected infection as previously reported [8,9,28,29]. As
7 the type of calorimeter used in this study allows parallel measurements of up to 48 samples, a
8 combination of test ampoules containing different growth media and conditions could be used to
9 further increase the sensitivity of the detection. Moreover, this strategy could be used as the
10 sample size of the presented method is relatively small. Indeed, patient samples could be
11 distributed on a number of ampoules containing different media. In such case, this combination
12 would allow the detection of anaerobes but also fastidious and slow-growing microorganisms
13 such as *Borrelia burgdorferi* sensu stricto or *Bartonella henselae* (data not shown), low-inoculum
14 or polymicrobial clinical samples.

15 We assume that the heat-flow curve will be the sum of heat production by each organism of the
16 mixture, depending on their inoculum, growth rate and potential interaction (competition) in the
17 same growth medium. At last, this approach has been developed to characterize *Mycobacterium*
18 *abscessus* susceptibility and can probably be establish for other and fastidious *Mycobacteriaceae*
19 species [30].

20 The calorimetry detection limit (TTD) of 10 μ W was defined previously [9]. In the present study
21 we observed, that even in cases where this value was not reached (detection considered as
22 negative), in most cases there was indeed an increasing heat flow, which was distinct from the
23 sterile control (background noise). A lower detection limit, as long as it is high enough to

1 discriminate from sterile samples, could further increase detection sensitivity by
2 microcalorimetry.

3 In summary, this calorimetric method is suitable to determine the optimal growth conditions for
4 fastidious and slow-growing microorganisms, e.g. anaerobes but also some fungi. Different
5 studies have also reported its ability to determine the efficacy of drugs alone or in combination
6 against planktonic and sessile strains optimizing the medium used [31–34]. Even though no
7 universal medium and growth condition could be found to allow a culture-based detection of the
8 multitude of different microorganisms causing infections, we demonstrate here that for specific
9 groups and species the optimization of growth conditions remains important to increase
10 sensitivity and decrease detection time of any culture based detection method.

11 In this study we employed the measurement of growth-related heat as a novel principle for
12 detection of anaerobes microorganisms in samples. With optimization, the sensitivity and
13 detection time can be further improved and have the potential to replace current microbiological
14 methods. Advantages of the calorimetric method compared to other techniques include its non-
15 invasive nature - measuring heat production without interfering with the microorganism, and no
16 need of clear solution – unlike spectrophotometric methods [35]. In addition, the calorimetric
17 method could have higher sensitivity compared to most standard methods [9,12]. At last, the
18 ampoule can be recovered easily to realize subcultures on different appropriate media to achieve
19 susceptibility testing, if necessary. This susceptibility testing can be also achieved using
20 microcalorimetry as previously reported [7,35,36]. We have not performed experiments with
21 mixed cultures, however, the heat-flow curve should represent the sum of heat production by
22 each organism of the mixture, depending on their inoculum, growth rate and interaction in the
23 same growth medium. Microcalorimetry may be, therefore, used for investigation of real-time
24 interaction between different microorganisms in mixed cultures.

1 For a future clinical use of this method, it is required to combine these results with the results of
2 previous studies and, of course, future studies using this new approach, to implement
3 microcalorimetry as a potential and useful tool to detect anaerobes or fastidious microorganisms
4 in small volumes of patient's body fluids.

5

6 **Conflicts of interest:**

7 All authors report no conflicts of interest relevant to this article.

8

9 **Acknowledgements:**

10 We thank Andrea Steinhuber for her comments to the manuscript. This work was supported by an
11 educational grant of the PRO-IMPLANT Foundation (<https://www.pro-implant.org>), a non-profit
12 organization supporting research, education, global networking, and care of patients with bone,
13 joint or implant-associated infections.

14

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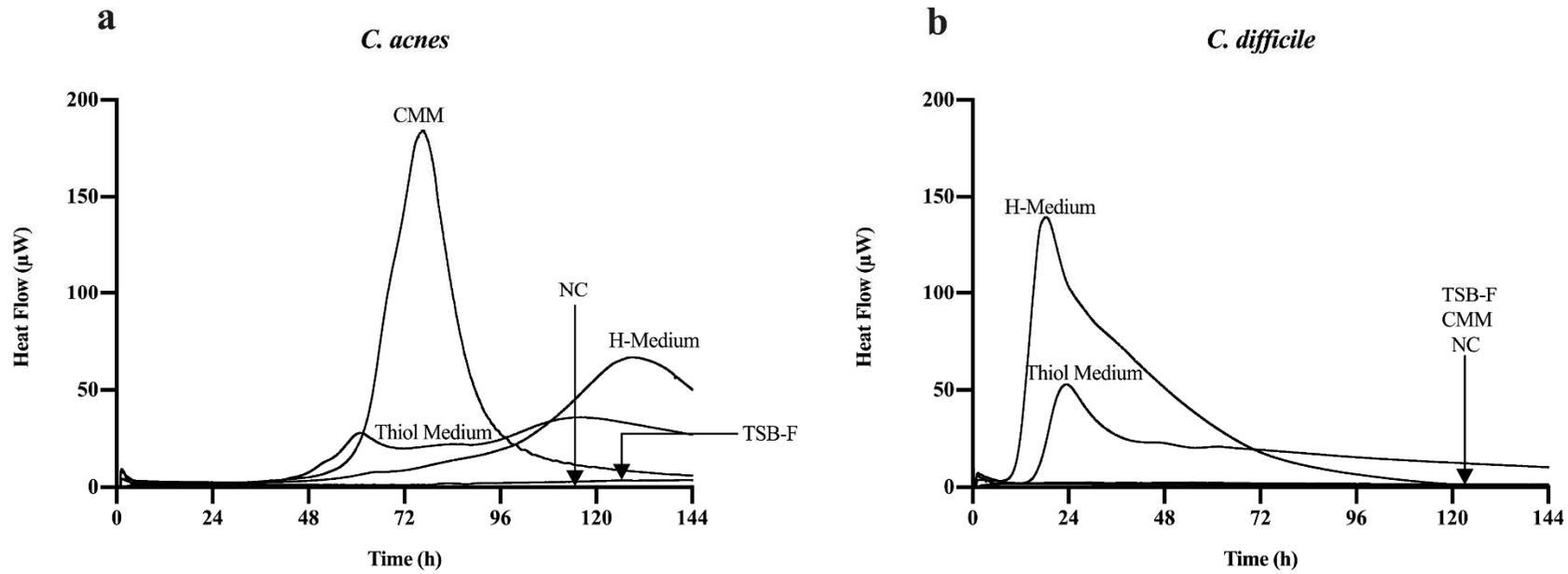


Fig1. Examples of representative heat flow curves obtained with the microcalorimeter over a 6-day period for *Cutibacterium acnes* (a) and *Clostridioides difficile* (b) detection using four different media Trypticase Soy Broth supplied with hemin and vitamin K (TSB-F), Cooked Meat Medium (CMM), Thioglycolate broth medium (Thiol Medium), and H-Medium, NC = negative control.

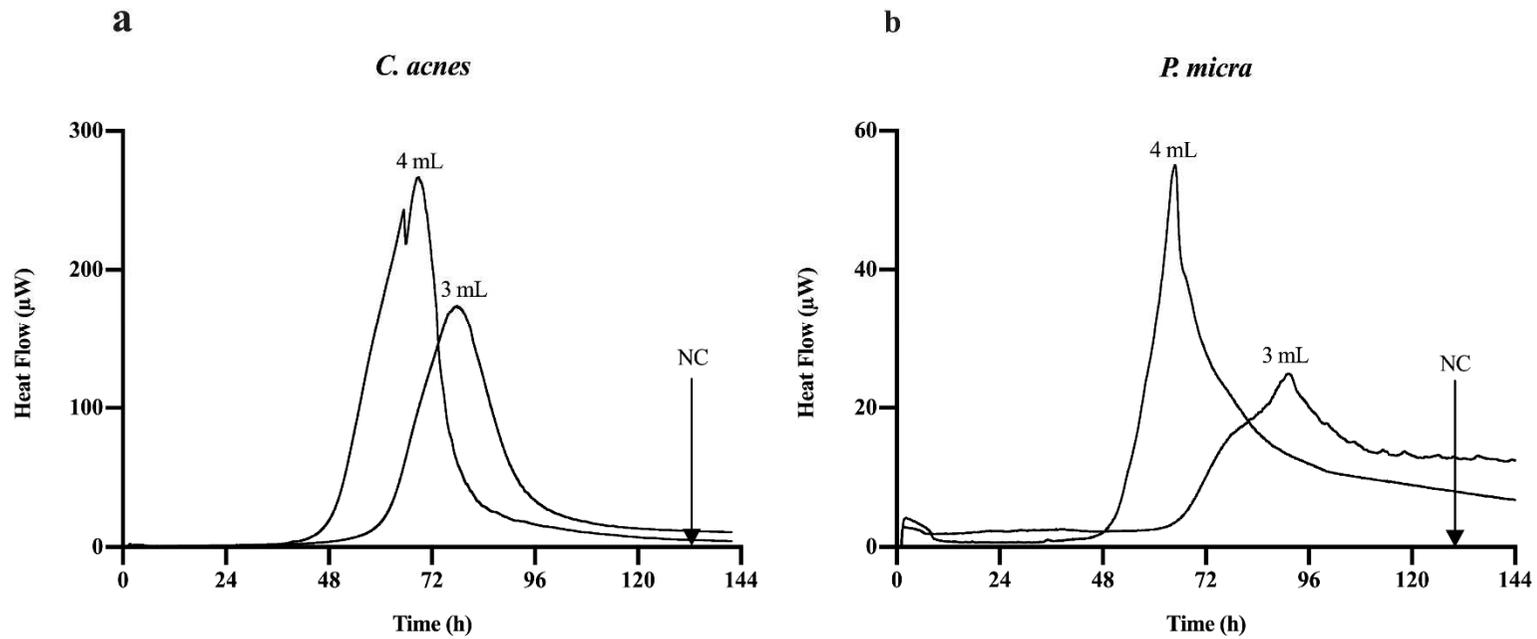


Fig2. Examples of representative heat flow curve differences (Time to detection) with the microcalorimeter during a 6-day period for *Cutibacterium acnes* (a) and *Parvimonas micra* (b) detection using 3 or 4 mL of Cooked Meat Medium (CMM) in the ampoule, NC = negative control.

1 **TABLE 1.** Influence of different media on microcalorimetric parameters for anaerobe detection. Numbers represent mean values \pm
 2 standard deviation of triplicate measurements.

Microorganism (Inoculum per ampoule in CFU)	TTD (h)				Peak (μ W)			
	TSB	CMM	Thio	H-Med	TSB	CMM	Thio	H-med
<i>C. acnes</i> (11,000)	-	55.5 \pm 2.2	49.8 \pm 1.9	75.4 \pm 2.2	3.8 \pm 1.9	184.4 \pm 9.8	36.1 \pm 2.0	66.8 \pm 3.9
<i>F. nucleatum</i> (260)	-	-	34.3 \pm 1.1	-	2.2 \pm 0.5	*	59.6 \pm 3.1	*
<i>F. magna</i> (665)	-	22.1 \pm 1.1	-	127.6 \pm 5.3	1.9 \pm 0.2	55.2 \pm 2.9	5.7 \pm 0.2	10.6 \pm 1.5
<i>P. micra</i> (3,300)	-	72.2 \pm 3.7	-	79.8 \pm 3.5	*	28.1 \pm 2.2	8.2 \pm 0.6	10.9 \pm 1.1
<i>B. fragilis</i> (4,000)	-	42.5 \pm 2.4	45.9 \pm 1.5	19.6 \pm 0.8	*	42.7 \pm 3.0	12.1 \pm 0.9	34.0 \pm 2.0
<i>A. odontolyticus</i> (4,700)	90.5 \pm 5.2	25.2 \pm 1.3	32.6 \pm 1.4	26.1 \pm 1.3	17.6 \pm 1.1	90.5 \pm 3.7	16.2 \pm 1.2	22.7 \pm 1.8
<i>C. difficile</i> (600)	-	-	16.4 \pm 0.8	10.5 \pm 0.7	*	*	52.9 \pm 2.0	140.3 \pm 8.4

3 Bold numbers represent the medium with the shortest TTD/ highest peak.

4 * No heat-flow peak measurable because the curve is sinking from the beginning.

5 - The heat limit of 10 μ W was not reached.

6 TSB = Trypticase Soy Broth supplied with hemin and Vitamin K

1 CMM = Cook Meat Medium

2 Thio = Thioglycolate

3 H-Med = H-Medium

4

1 **TABLE 2.** Influence of different volumes of medium on microcalorimetric parameters for anaerobe detection. Numbers represent
 2 mean values \pm standard deviation of triplicate measurements.

Microorganism (Inoculum per ampoule in CFU)	Medium	TTD (h)		Peak (μ W)	
		3 mL	4 mL	3 mL	4 mL
<i>C. acnes</i> (2,200)	CMM	32.5 \pm 1.9	45.2 \pm 1.6	173.8 \pm 5.1	266.7 \pm 8.8
<i>F. nucleatum</i> (30)	Thio	50.3 \pm 1.5	35.4 \pm 2.1	26.9 \pm 1.1	92.8 \pm 3.6
<i>F. magna</i> (110)	CMM	19.1 \pm 1.1	15.9 \pm 1.0	49.5 \pm 2.6	102.3 \pm 6.0
<i>P. micra</i> (1,400)	CMM	71.7 \pm 2.0	53.3 \pm 0.8	24.8 \pm 1.8	55.0 \pm 2.2
<i>B. fragilis</i> (7,400)	H-med	17.7 \pm 0.9	14.2 \pm 1.2	39.6 \pm 2.2	87.7 \pm 2.5
<i>A. odontolyticus</i> (10,000)	CMM	22.8 \pm 1.6	22.5 \pm 1.9	88.7 \pm 4.1	83.7 \pm 2.1
<i>C. difficile</i> (815)	H-med	16.6 \pm 0.3	16.4 \pm 1.6	97.2 \pm 3.5	139.5 \pm 5.9

3

4 CMM = Cook Meat Medium

5 Thio = Thioglycolate

6 H-Med = H-Medium