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Freezing preservation procedure of caecal inoculum for microbial fermentation studies in pigs

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Abstract

Aim of study: To define freezing conditions that preserve fermentative capacity of microbial inoculum for *in vitro* studies in pigs.

Material and methods: Caecal contents from three slaughtered pigs were obtained for being used as inoculum. Part of it was immediately frozen in liquid N and stored at -80ºC, whereas the rest was directly used as fermentation inocula. Incubation substrate was pre-digested in pepsin and pancreatin to simulate the processes occurring before the caecum. Pre-digested substrate was incubated alone or supplemented by three additives consisting of two commercial additives based on essential oils mixtures (CRINA-TEP and CRINA-TMEC) and riboflavin. Gas production at 2, 4, 6, 8, 10, and 12 h, and methane, short chain fatty acids (SCFA), and ammonia concentration at 6 h and 12 h were recorded.

Main results: No differences (p>0.05) were recorded along the 12 h incubation between both preservation methods of inocula regarding gas production, methane or total SCFA or their molar proportions. Only a trend for a higher ammonia concentration was detected with frozen than fresh inocula (p=0.062). Although not a main objective of the paper, gas production from the substrate alone (control) was lower than with riboflavin from 8 h onwards, and with CRINA-TEP from 4 to 10 h incubation (p>0.05).

Research highlights: Caecal inoculum from pigs for *in vitro* fermentation studies can be preserved by freezing, provided that freezing and thawing processes are carried out under favorable conditions, especially in terms of time and temperature.

Additional key words: inoculum preservation; gas production; *in vitro*; monogastrics.

Abbreviations used: BCFA (branched-chain volatile fatty acids); DM (dry matter); OM (organic matter); SCFA (short chain fatty acids).

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Introduction

In vitro digestion techniques for simulating physiological processes are widely extended in animal nutrition. Generally, enzymatic approaches have been applied for digestion gastric and small intestine in studies for monogastrics (Boisen & Fernandez 1995, 1997; Yegani *et al*., 2013), with results obtained being similar and highly correlated with *in vivo* feeding studies (Chiang *et al*., 2008). However, the enzyme approach does not fit so well for mi-

crobial fermentation studies, since used enzymes are commonly from aerobic fungi, and do not necessarily act over the substrate at the same level, and conditions for their optimal activity does not necessarily match with environmental conditions.

Previous efforts have been published to adapt the gas production technique to studies of hindgut fermentation in monogastric animals, such as pigs (Fondevila *et al*., 2002; Williams *et al*., 2005), horses (Lowman *et al*., 1999) or rabbits (Calabró *et al*., 1999; Rodríguez-Romero *et al*., 2011). Special consideration must be paid to the fact that incubated substrate must be pre-digested simulating gastric and small intestine digestion to accurately simulate characteristics of what arrives to the caecum (Fondevila *et al*., 2002).

As studied for ruminants, a major issue in the application of the gas production technique is the restricted viability of the microbial inoculum, that force to use it fresh, immediately after extraction (Mould *et al*., 2005). The immediate use of inoculum (fresh) in *in vitro* fermentation studies is recommended for most procedures (Menke & Steingass, 1988; Theodorou *et al*., 1994). However, this is often difficult to ensure in practical situations. Alternatively, refrigeration of microbial inoculum has been proposed, provided that inoculum is maintained at 4-6ºC for less than 6 h (Robinson *et al*., 1999; Hervás *et al*., 2005). Prates *et al*. (2010) demonstrated that the freezing process of microbial inoculum, either in liquid N (-196ºC) or even in a regular freezer (-20ºC) do not noticeably affect fermentation activity of the microbial community, either quantitative or qualitatively, respect to the conventional use of fresh inoculum. Thus, preservation of digesta contents for being used in subsequent *in vitro* studies is considered an important issue that improves the advantages of *in vitro* methods. The response to freezing microbial inoculum has been previously tested for ruminants, but it has not yet been validated for fermentation studies in monogastrics, as it is the objective of the present study. Methods were tested with a common substrate supplemented with different additives aiming to reach a range of *in vitro* microbial fermentative activity.

Material and methods

Experimental procedures

All animal procedures were carried out under the Project License PI54/18NE, approved by the Ethic Committee for Animal Experimentation from the University of Zaragoza, Spain. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD118/2021, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Three pigs (on average 23.9 ± 1.01 kg) were given a commercial feed without pre/probiotics (P08-GS, Agroveco, Casetas, Spain) for 30 days for being used as donor of inoculum. This feed was based on a mixture of barley, wheat, maize, soybean meal and palm kernel cake (151 g crude protein, 40 g crude fibre, 8.9 g lysine per kg fresh basis). At the end of the diet adaptation period pigs weighed on average 43.3 kg ± 3.56 kg. Then, animals were slaughtered after CO_2 stunning, and the caecal contents were extracted. After recording pH with a glass electrode pH meter (CRISON 507, CRISON, Barcelona, Spain), part of caecal contents was divided into 18 mL aliquots, dispensed in 20 mL polypropylene tubes (110 × 16 mm), immediately frozen in liquid N and thereafter stored at -80ºC as in Prates *et al*. (2010) for later use as inoculum for *in vitro* gas production studies. The rest of caecal contents were immediately transferred to the lab in thermos flasks and maintained at 39ºC under a CO_2 stream before being directly used as fermentation inocula less than one hour later.

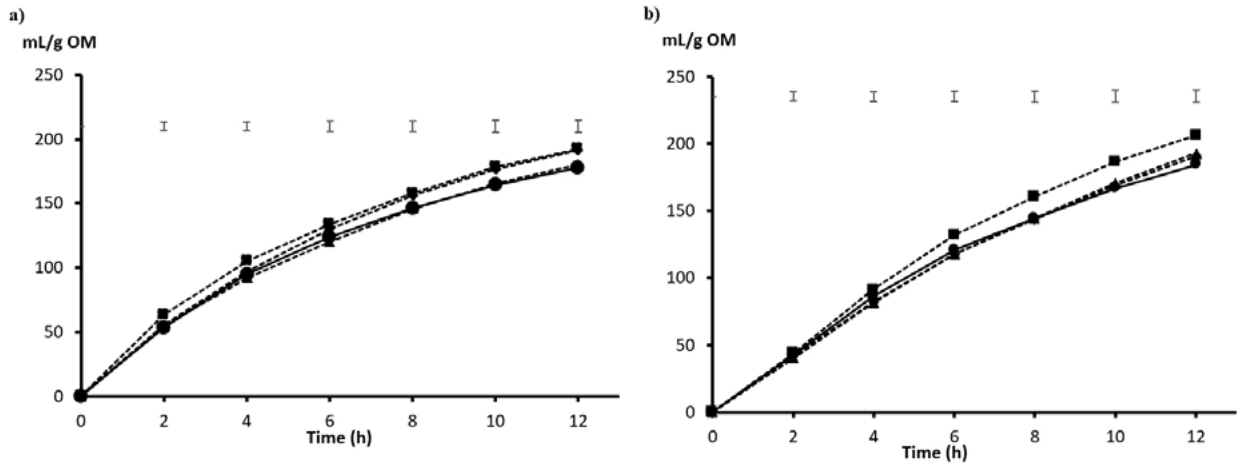

Figure 1. Average gas production pattern (mL/g organic matter, OM) from substrate incubated unsupplemented (●, continuous line) or added with CRINA-TEP (80 mg/kg, ◆), CRINA-TMEC (100 mg/kg, ▲) or riboflavin (100 mg/kg, ■), with fresh (a) or frozen (b) inocula from the caecum of pigs. Upper bars show standard error of means.

Table 1. Methane production (volume, mL/g organic matter, and proportion in total gas) and average fermentation parameters (concentration of total short chain fatty acids (SCFA, mM), and molar proportions of the main SCFA, and ammonia concentration, mM) according to the preservation conditions of the inocula, at 6 and 12 h incubation.

	6 h				12 h			
	Fresh	**Frozen**	**sem**	***p*-value**	**Fresh**	**Frozen**	**sem**	***p*-value**
Methane:								
Volume	1.060	1.223	0.4527	0.82	2.117	2.872	1.1015	0.68
Proportion	0.009	0.009	0.0015	0.98	0.022	0.017	0.0046	0.73
Ammonia	12.73	17.83	0.940	0.062	17.30	18.50	0.647	0.32
Total SCFA	46.18	41.89	2.684	0.54	51.27	47.64	4.246	0.53
Acetate	0.425	0.461	0.0229	0.30	0.372	0.387	0.0233	0.67
Propionate	0.214	0.220	0.0177	0.80	0.223	0.229	0.0090	0.74
Butyrate	0.129	0.102	0.0099	0.13	0.157	0.147	0.0113	0.53
Valerate	0.019	0.009	0.0039	0.16	0.026	0.020	0.0034	0.52
BCFA	0.0063	0.0041	0.0020	0.20	0.011	0.009	0.0012	0.20

BCFA: sum of isobutyrate and isovalerate proportions. sem: standard error of means. Within rows, letters indicate treatment differences ($p<0.05$).

As incubation substrate, the same feed given to pigs was used, being previously pre-digested to simulate the digestive processes occurring before the caecum. Pre-digestion was carried out by successively incubating feed (100 g) in pepsin-HCl (Sigma P-7000, 25 mg/mL) and pancreatin (Sigma P-1750, 100 mg/mL), according to the procedure of Boisen & Fernández (1997). Once predigested, substrate was dried at 60ºC for 48 h and divided into 4 parts for being incubated alone (as a control) or supplemented by three eubiotics from DSM company, two different mixtures of essential oils (CRINA-TEP, at 80 mg/kg and CRINA-TMEC, at 100 mg/kg) and riboflavin (100 mg/kg), aiming to promote different responses in microbial activity. Doses of additives were established according to the recommendations from the manufacturer.

The microbial fermentation pattern of caecal contents was studied *in vitro* by the gas production technique (Theodorou *et al*., 1994). An amount of 0.8 g was incubated on each bottle. Firstly, triplicate glass bottles (110 mL total volume) for each treatment were filled with 56 mL incubation solution. Caecal contents of each of the 3 pigs for each treatment were mixed 2:1 with the incubation solution under CO_2 atmosphere and homogenized for few minutes with waring blender. Then, 24 mL of this mixture were added to each bottle to complete the 80 mL total liquid volume, thus caecal inoculum resulted in a 0.20 of total incubation solution. In addition, triplicate bottles without substrate were also included as blanks. Bottles were sealed under a CO_2 stream and incubated at 38ºC in a water bath for 12 h. Note that the procedure of the *in vitro* gas production was the same for both fresh inocula and frozen inocula, with the only difference that the frozen samples of caecal contents were previously maintained at 38ºC in a water bath for 2-3 min for thawing.

Pressure was recorded every two hours throughout the incubation period, by means of an HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy). Readings were converted into volume by a pre-established linear regression equation between the pressure recorded in the same bottles under the same conditions and known air volumes (n= 103; R^2= 0.996). The gas volume recorded for each incubation time was expressed per unit of incubated organic matter (OM). Methane (CH_4) concentration was measured on every 6 h interval from 6 mL aliquots of the gas produced that was sampled after pressure recording on vacutainer tubes with a common pre-established vacuum. At 6 and 12 h incubation samples were collected and either acidified 1:1 with 0.1 mmol L^{-1} HCl or added to 0.5 mL of a deproteinizing mixture of 0.5 mmol L^{-1} PO_4H_3 with 2 mg/mL 4-methyl valeric acid, and were stored at -20ºC for determination of ammonia and short chain fatty acid (SCFA) concentration, respectively.

Chemical and statistical analysis

Substrates were analysed for dry matter (DM) and OM according to the AOAC (2005) procedures (methods ref. 934.01 and 942.05, respectively). For comparison purposes, the four pre-digested substrates were incubated with Viscozyme (Sigma-Aldrich V2010) in three runs of duplicate flasks (Boisen & Fernández, 1997), to simulate the last step of the enzymatic digestion procedure.

Methane concentration was measured in an Agilent 6890 apparatus equipped with a FID detector and a capillary column (HP-1, 30 m × 535 µm id × 1.5 µm film thickness), calibrated with a 10% CH_4 standard, with a flux

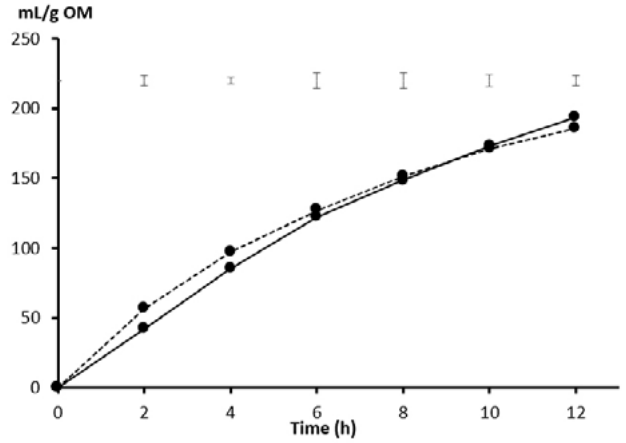

Figure 2. Average gas production pattern (mL/g organic matter, OM) of fresh (dotted line) or frozen (continuous line) inocula from the caecum of pigs given the experimental treatments. Upper bars show standard error of means.

of 2 mL/min at 250ºC. Injection was on split mode, with He as carrier gas. Frozen samples of incubation medium were thawed and centrifuged at 13,000 g for 15 min at 4 ºC, and the concentration of individual SCFA in incubated medium was determined by gas chromatography on an Agilent 6890 apparatus (Agilent Technologies España S.L., Madrid) equipped with a FID detector and a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m × 530 µm id × 1 µm film thickness). Injection was on column, with He as carrier gas and a temperature ramp from 80 to 165 to 230 ºC. Concentration of ammonia was determined colorimetrically following the procedure from Chaney & Marbach (1962).

Results were contrasted statistically by ANOVA using the Statistix 10 package (Analytical Software, 2010) following a split-plot design according to the model:

$$Y = \mu + Pi + Mj + PMij + Ak + MAjk + \varepsilon l(ijk),$$

with the preservation method as the main plot (*Mi*, n=2) and the donor pig as a block (*Pi*, n=3) that were tested against the interaction *PMij*, and the dietary additive as the subplot (*Ak*, n=4) and the interaction *MAjk* (n=6) that were contrasted against the residual $\varepsilon l(ijk)$. The average of three bottles for each treatment incubated with the same inoculum (incubation run) was considered as the experimental unit. In case of detecting significant treatment effects, differences were contrasted by the Tukey t-test at $p<0.05$. A trend to significance was considered when $p<0.10$.

Results

Addition of riboflavin to the substrate increased in a 0.09 proportion (14 to 18 mL gas/g OM) the volume of gas produced respect to control from 8 h onwards, and it was also higher that with CRINA-TEP from 4 to 10 h incubation ($p<0.05$). No effect of the interaction additive × preservation method was recorded ($p>0.05$), but since the major objective of this work was to compare among inocula, the gas production pattern of incubated substrates with fresh or frozen inocula is shown separately in Figure 1.

Comparison of gas production of fresh and frozen caecal inocula is shown in Figure 2. The gas production pattern from samples incubated with liquid N frozen inoculum did not differ from that with fresh inoculum ($p>0.10$), and only a trend ($p=0.081$) for a lower volume with the frozen inoculum was noticed at 4 h incubation. A significant effect of the additive included on the volume of gas produced was detected from 4 h onwards ($p<0.05$). In any case, the interaction inoculum x additive was also not significant ($p>0.05$) throughout the incubation period.

As it is showed by the ANOVA, the proportion of the effect of donor pigs to overall variation in gas production ranged from 0.32 to 0.58. Thus, the coefficient of variation of the donor pig × preservation in the split-plot analysis was relatively low (ranging 0.066-0.152 from 4 to 12 h), highlighting the homogeneity in the response to the inoculum preservation.

No effect of the dietary additive was recorded on methane production, ammonia or SCFA concentration, nor on the molar SCFA proportions along the incubation ($p>0.10$). Since the interaction additive × preservation method also resulted non-significant ($p>0.10$), Table 1 only shows average means for the preservation methods for these parameters at 6 and 12 h incubation. When comparing preservation methods, no differences ($p>0.05$) were recorded on CH_4 production along the 12 h incubation between fresh and frozen inocula, either in terms of volume produced or on its proportion of total gas. The ammonia concentration after 6 h incubation tended ($p=0.062$) to be higher with frozen than fresh inocula, but this trend disappeared at 12 h ($p>0.10$). Total SCFA concentration or their molar proportions were not affected by the preservation method of inoculum at 6 nor at 12 h incubation ($p>0.05$).

Discussion

Considering the location of the fermentation site in the digestive tract of monogastric animals, as well as the fact that feeds for pigs and poultry are mostly compound of digestible carbohydrates and proteins that disappear in the first part of the digestive tract, pre-digestion with gastric and small intestine enzymes is a necessary step for the evaluation of hindgut fermentation in these animals (Fondevila *et al*., 2002). In our experiment, the pepsin-pancreatin treatment digested, on average, a proportion of 0.80 of initial feed DM, which should obviously affect the composition of incubated substrate (not analysed). As a further reference, simulation of polysaccharide digestion with enzymes (Viscozyme) as the last step of the digestion enzy-

matic process (Boisen & Fernández, 1997) digested 0.453 ± 0.115, but it accounted for only 0.051 ± 0.0138 of total feed DM digestion.

The freezing at -70ºC has been applied as routine procedure for preserving pure bacterial cultures in microbiological studies, either with or without cryoprotectants (Gibson & Khoury, 1986; Malik, 1991; Perry, 1998), despite Fonseca *et al*. (2001) suggested some selective effect depending on the microbial membrane structure. *In vitro* fermentation studies in which freezing failed to maintain microbial fermentative capacity (Robinson *et al*., 1999; Hervás *et al*., 2005; Denek *et al*., 2010) used large batches of inoculum, and did not consider the time of freezing and thawing in the preparation process. In this regard, a fast processing is mandatory for ensuring the maintenance of cell viability and fermentative capacity (Malik, 1991; Perry, 1998). Dispensing in tubes with high surface to volume ratio should ensure a fast processing, and therefore the viability of the microbial community of the inoculum.

Other more recent approaches in ruminant fermentation studies have assayed the ability of frozen inoculum, either with or without cryoprotectants, to maintain similar fermentation characteristics than fresh inoculum. Chaudhry & Mohamed (2012) obtained a high correlation among fresh and frozen (-20ºC) inoculum in *in vitro* DM degradation pattern but results were of 0.20 lower magnitude. Belanche *et al*. (2019) froze small volumes of inoculum (35 mL) at -80ºC using DMSO as cryoprotectant, and Spanghero *et al*. (2019) froze 250-300 mL aliquots at -80ºC without cryoprotectant, but both works detected a lower gas production than the fresh inoculum. Despite the differences in freezing temperatures, it can be suspected that there is a common methodological aspect in all these efforts: either the freezing or the thawing periods, or both, were carried out relatively slowly. This might be due because of large volumes may take delay freezing and thawing, thus maintaining exposure of microbial community to low temperatures for an extended period of time, thus reducing its viability, as it could be the case of Robinson *et al*. (1999), Hervás *et al*. (2005), Chaudhry & Mohamed (2012) or Spanghero *et al*. (2019). In other sense, the thawing process lasted for less than one minute in Denek *et al.* (2010), 5 minutes in Belanche *et al*. (2019), 2 h in Spanghero *et al*. (2019) and 12 h in Chaudhry & Mohamed (2012). Further, in the case of Denek *et al*. (2010) and Belanche *et al*. (2019), where small amounts of inoculum were frozen in liquid N, DMSO was used as cryoprotectant, but this was not removed from the medium and its toxicity at culture temperatures (Hubálek, 2003) might probably affect microbiota when became stable at optimum temperature. This reinforces the work from Prates *et al*. (2010) who hypothesised that the length of the freezing and thawing processes is critical, and that a cryopreservant is not really necessary for studies of microbial fermentation. Further, the use of glycerol as cryoprotectant enhanced later fermentation as it was used as energy source for microorganisms. Besides, recovery of frozen culturable rumen bacteria is in general adequate (Zehavi *et al*., 2018) and activity of other microbial types such as protozoa is minimal under conventional *in vitro* media (Dehority, 2008). In other way, the freezing temperature does not seem to be determinant: Prates *et al.* (2010) did not observe major differences between freezing at -20ºC or in liquid N, provided that inoculum was dispensed in small tubes for maximising the surface to volume ratio. In the present experiment, the same protocol of Prates *et al*. (2010) for preserving and activating the inoculum was followed for pig caecal contents, and the obtained results confirm the positive responses obtained by those authors.

Result from our study showed that both types of inoculum behaved similarly in terms of rate and magnitude of substrate fermentation. Further, individual responses for each donor animal also showed a similar fermentative pattern. Values of fermentation parameters did not differ among inocula, as well as for ammonia concentration at 12 h incubation. It is worth considering that no differences on molar volatile fatty acids proportions were recorded by Prates *et al*. (2010) despite the processing method of the inocula (fresh, refrigerated at 6ºC for 4 h, frozen at -20ºC or frozen in liquid N) after 6 or 24 h incubation.

In a recent study (Ma *et al*., 2022) it was observed that, despite overall microbial diversity was not affected by freezing rumen inoculum, it affected the abundance of certain genera of bacteria, and *Ruminococcus* increasing and *Christensenellaceae* R7 tending (p=0.09) to decrease, the latter being involved in butyrate production (Morotomi *et al*., 2012). However, results from Ma *et al*. (2022) do not support a selective positive effect on protein degradation bacteria that should justify the higher ammonia concentration observed in the medium with frozen inoculum. In line with the above discussion, it is worth considering that, in their work inoculum was frozen in liquid N, but thawing was carried out at 37ºC for 10 min. In any case, further microbiological analyses are necessary to determine these effects on caecal microbial fermentation.

In conclusion, despite the mentioned approaches in *in vitro* studies for ruminants, to our knowledge this has not been boarded with inoculum from monogastric animals. Our results confirm that microbial inoculum can be dispensed in tubes, frozen in liquid N and stored at -80ºC without compromising its *in vitro* fermentative activity, either quantitatively or qualitatively.

Authors' contributions

Conceptualization: M. Fondevila, Z. Amanzougarene, E. Pérez-Calvo.
Data curation: M. Fondevila, Z. Amanzougarene.
Formal analysis: M. Fondevila, Z. Amanzougarene.
Funding acquisition: E. Pérez-Calvo.
Investigation: Z. Amanzougarene.

Methodology: M. Fondevila, Z. Amanzougarene.
Project administration: M. Fondevila.
Supervision: M. Fondevila.
Writing – original draft: M. Fondevila, Z. Amanzougarene, E. Pérez-Calvo.
Writing – review & editing: M. Fondevila, Z. Amanzougarene, E. Pérez-Calvo.

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