Short communication: Effect of various dietary regimens on oral challenge with *Mycobacterium avium* subsp. *paratuberculosis* in a rabbit model

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Abstract

Rabbits are susceptible to infection by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in both wildlife and experimental conditions. Under the hypotheses that nutrient balance might influence the establishment of infection, we designed an experiment where MAP intestinal colonization was assessed under three dietary regimens: high fiber, high protein, and regular diet in New Zealand white rabbits submitted to oral challenge with MAP.

Lowest weight gain ($F=5.17$, $p=0.024$), higher tissue culture positivity rates ($\chi^2=7.43$, $p=0.024$) and especially extended MAP-compatible lesions ($F=5.78$, $p=0.017$) were detected in the regular diet.

Taken altogether, results indicate that paratuberculosis infection was achieved affecting mostly regular diet animals and showing that dietary changes may modulate the course of the infection.

Key words: *Mycobacterium avium* sbsp. *paratuberculosis*, Johne's Disease, infection, rabbit, animal model, diet.

Mycobacterium avium subspecies *paratuberculosis* (MAP) is responsible for a chronic granulomatous enteritis named paratuberculosis (PTB), distributed worldwide (Kennedy and Benedictus, 2001, Manning and Collins, 2001). Although PTB has been historically linked to domestic ruminants there is evidence that wild-life species, both ruminant (Chiodini and Van Kruiningen, 1983) and non-ruminant (Beard et al., 2001a, Greig et al., 1997) are susceptible to natural infection. Association of MAP with Crohn's disease makes PTB a human health concern, as well (Hermon-Taylor, 2001).

The pathogenic mechanisms of PTB have not been fully elucidated probably due to the lack of an appropriate laboratory animal model and the slow infection characteristics including the lengthy incubation period of the disease (Juste, 2012).

Rabbits are a convenient laboratory species and natural PTB infections of wild rabbits (Angus, 1990, Beard et al., 2001b, Greig et al., 1997) have been described. Intestinal lesions consistent with PTB (Harding, 1959, Mokresh and Butler, 1990, Mokresh et al., 1989, Vaughan et al., 2005) as well as diarrhoea (Mokresh and Butler, 1990, Mokresh et al., 1989) and fecal shedding (Mokresh et al., 1989) have been reported previously in rabbits orally inoculated with MAP in laboratory conditions. Susceptibility to MAP in both experimental (Harding, 1959, Mokresh and Butler, 1990, Mokresh et al., 1989, Vaughan et al., 2005) and natural (Beard et al., 2001b, Greig et al., 1997) infection conditions suggests rabbits may be a suitable model for PTB.

Dietary changes have been shown to have an effect on infectious diseases caused by bacteria (Fox and Wang, 2014, Zumbrun et al., 2013). We hypothesized that dietary changes could influence MAP infection. Therefore, the

aim of this study was to evaluate the effects of diet shifts during MAP challenge to gather information that might aid further investigation regarding the potential use of rabbits as a PTB model. To test the hypothesis, three different diet conditions: regular rabbit (R), high fiber (HF), high protein (HP) were tested simultaneous to oral challenge with MAP strain K10. Infection progression was evaluated and monitored by MAP isolation on solid media and quantitative polymerase chain reaction (qPCR) of feces and tissues, as well as by histopathological examination of tissues.

MAP strain K10 was cultured on Middlebrook 7H9 (7H9) liquid media supplemented with OADC and mycobactin J (MJ) for 4 weeks at 37 +/-1ºC. Bacterial concentration was adjusted in PBS by turbidometry. Colony forming unit (CFU) counts were confirmed on 7H9 OADC MJ agar plates. The final challenging dose was $4x10^8$ CFU of MAP.

New Zealand rabbits were purchased from an accredited animal dealer (Granja San Bernardo, Navarra) arriving at the animal facilities at an age of 6 weeks (1.5 kg). After a two week adaptation period fed with weaning pellets, animals were tattooed for identification and started feeding with diet R for 10 days. They were then divided into three diet groups: R (n=5), HF (n=5) and HP (n=5). Diet compositions are detailed on supplementary Table 1. Four days after commencing these diets, weight was recorded and feces were collected from all animals ($S_{0,}$ day 0). On three consecutive days (days 1, 2 and 3) animals were orally administered a single challenging dose per day. Feces were collected on day 3 (S_{0A}) to check MAP pass through the digestive tract. Also, from day 3 on, all animals were fed diet R until the end of the experiment (day 114).

Monitorization consisted of weight recording along with feces collection twice a month (S_1-S_8).

The study was designed following European, National and Regional Law and Ethics Committee regulations and it underwent ethical review and approval by NEIKER´s Animal Care and Use Committee and by the Agriculture Department (PARAMOD-6278-BFA).

At the endpoint, animals were injected with xylazine (5 mg/kg) and ketamine (35 mg/kg) intramuscularly for sedation. Then pentobarbital was injected intracardiacly.

For microbiological examination, samples from ileum, jejunum, sacculus rotundus (SR), ileocecal junction, vermiform appendix (VA), liver, spleen, muscle, tonsils and cecal contents were collected and stored at -20ºC. For histopathological examination, all previously mentioned samples except for cecal contents were taken and processed as described by (Vazquez et al., 2013) .

Slides were examined under the microscope and granuloma extension was measured using Image J software (http://imagej.nih.gov/ij/) (Schneider et al., 2012) on two micrographs of each section. The lesion index was calculated as the total area of granulomatous lesion divided by the total area of the micrograph.

Two grams of freshly collected feces were cultured on solid Herrold Egg Yolk Medium (HEYM) as described by (Aduriz et al., 1995). For culture on 7H9 OADC MJ penicillin, anfotericin and cloramphenicol, the decontaminated suspension was washed twice with sterile water (2885 x g during 10 min). Four drops/ tube of the final pellet suspended in 2 ml of water were seeded.

For tissue culture, tonsils, spleen, liver, and muscle were spliced into tiny pieces whereas VA, SR, ileum and jejunum were scraped for mucosa. Previously mentioned samples along with cecal content were weighed and identical protocol as for feces was followed.

DNA extraction from feces was done following manufacturer's instructions of DNA Extract-VK (Vacunek S.L.). For tissues, brief modifications described by (Arrazuria et al., 2015) were performed. In both cases, extracted DNA was stored at -20ºC for PCR analysis.

MAP detection was performed following a MAP F57 PCR (Schonenbrucher et al., 2008). Samples yielding C_T values equal or below 37 for F57 probe were considered positive. In these cases, MAP genomic equivalents (GE) were estimated by ParaTB Kuanti-VK qPCR (Vacunek, S.L.) as described by (Elguezabal et al., 2011).

For weight, fecal PCR MAP tissue PCR and lesion analysis ANOVA approach based on summary measures was used (weight gain: the difference between S_8 weight and S_0 weight, total fecal shedding: total GE/g of MAP detected in feces by each animal during the experiment, total MAP in tissues: the sum of MAP GE/g in all examined tissues, total lesion index (TLI): sum of the lesion extension in all examined tissues. For dichotomous variables such as tissue culture, logistic regression was used taking R as the reference category. For weight and fecal PCR, analysis of repeated measurements was done by mixed-effect regression, including individuals as random-effect. Multiple Correspondence Analysis (MCA) was used as a multivariate exploratory analysis to detect and graphically represent underlying structures in the data (Benzécri, 1969). All the in vivo and post mortem measurements were included

141 in the MCA as categorical versions of the original variables. All statistical
142 analyses were performed using R statistical software (3.1.0) and significance of
143 the differences among groups for all variables were stated at $p < 0.05$.
144 During the in vivo follow up no overt clinical signs were observed and weight
145 loss between samplings was minimal and exceptional. This was expected since
146 weight loss has shown to be rare in long term experiments (Mokresh et al.,
147 1989, Vaughan et al., 2005).Diet R animals gained less weight than animals
148 that had been on the other two diets during challenge and significant differences
149 were observed both when weight gain among groups was analyzed ($F=5.17$,
150 $p=0.024$). Moreover, considering all the measurements over time, diet R has
151 significantly less weight than HF (Supplementary Table 2)
152 MAP passed through the digestive tract demonstrated by culture and q-PCR of
153 sampling $S0_A$ feces (Table 1), with no significant differences in bacterial load
154 among diet groups suggesting that challenge was achieved equally in all
155 animals.
156 No episodes of diarrhoea were observed and fecal culture was positive only in
157 sampling $S0_A$ in 66.6% of the animals being negative in all cases thereafter. In
158 previous experimental infections, MAP was either not isolated from feces
159 (Mokresh and Butler, 1990, Vaughan et al., 2005) or isolated from 30.7% of
160 infected animals (Mokresh et al., 1989). Unsuccessful fecal isolation could be
161 due to low detection limit by culture, light shedding or low sampling frequency.
162 qPCR showed higher detection capacity since all animals were positive in $S0_A$
163 and MAP shedders were detected throughout experiment samplings. Total MAP
164 shedding tended to be higher in diet group R although significant differences
165 were not detected.

Gross lesions consisting in pale-white reactive spots were detected in the SR and VA in diet R (40%) and diet HF (20%) animals contrary to previous studies where macroscopic lesions were not reported (Mokresh and Butler, 1990, Mokresh et al., 1989, Vaughan et al., 2005).

Microscopically, animals presented granulomatous infiltrates in the SR and VA located in the follicular and/or interfollicular regions depending on the diet. Well demarcated granulomas with a huge variability in size were detected (Supplementary Figure 1 A, B and C). These findings are consistent with PTB infection and could be equivalent to focal and multifocal lesions detected in subclinically infected goats (Corpa et al., 2000) or sheep (Pérez et al., 1996). AFB were detected in SR of only one rabbit from diet group R indicating a low bacterial colonization, a dormancy-related loss of acid-fastness (Zhang, 2004) or too short duration of the experimental trail.

The TLI was higher in diet group R (0.550 +/- 0.359) compared to diet HF (0.100 +/- 0.068) and diet HP (0.196 +/- 0.108) showing significant differences ($F=5.78$, $p=0.017$) (Supplemental Figure 1 D), suggesting diet R could favour MAP tissue reaction or that diets HF and HP were able to limit lesion extension.

Mucosa from VA, SR, ileum and jejunum was MAP positive by culture (Table 1). Tissue locations are in agreement with previous works showing MAP culture positive results for VA (Mokresh et al., 1989, Vaughan et al., 2005) and SR and ileum (Mokresh et al., 1989). Diet group R showed a higher MAP culture positivity rate (60%) ($\chi^2=7.43$, $p=0.024$). Differences between diet group R and HF were observed in both VA ($p=0.035$) and SR ($p=0.008$), and differences between diet R and diet HP in SR ($p=0.008$). Bacterial load measured by qPCR

was variable among specimens and individual animals showing no significant differences among groups.

MCA analysis gave a picture of the infection outcome, by explaining 62% of the variability in the measurements (Figure 1). The resulting two dimensional map clearly shows that diet R animals are correlated to higher rates of infection since most positive results and high rates were concentrated on the right side of the graph where 80% of the animals with diet R appeared, whereas negative results and low indexes were in the left side, where 80% of the animals from the other diets were located.

In conclusion Diet R performed best at aiding infection in the assayed conditions and the two diet changes could be modifying the course of infection in a way that we cannot explain at the moment. These results suggest that there is a strong interaction between diet and exposure to MAP that should be further investigated.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. Map created by the first two components derived from the multiple correspondence analysis.

Supplemental Figure 1. Histopathological findings. Hematoxylin and eosin-stained section micrographs of the sacculus rotundus of a rabbit from: (A) diet group R showing well-demarcated large granulomas containing macrophages as the main cellular population in the follicles and interfollicular region (100X), (B) diet group HF showing a few small granulomas (200X), (C) diet group HP showing medium size granulomas in the interfollicular region (200X). (D) Total Lesion Index calculated as the sum of the area of lesion divided by the area of the micrograph of all examined specimen sections. The solid lines show the mean values.

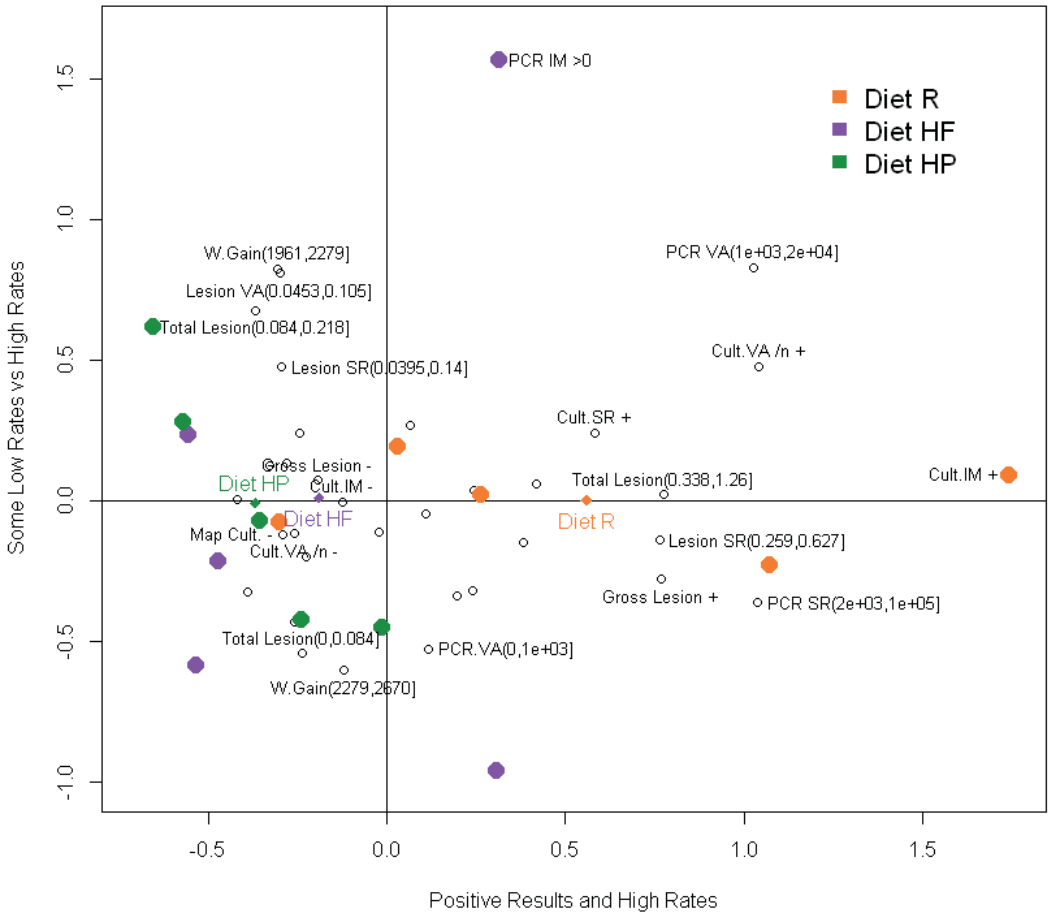
Table

Table 1. Microbiological findings in feces through in vivo follow up and in post mortem tissues.

Diet	Animal	MAP levels by qPCR (GE/g)									MAP culture		
		Feces						Tissues			Tissues		
		$S0_A$	S1	S4	S5	S6	S8	VA	SR	IM	VA	SR	IM
	NT	$1,45\ 10^5$	0	$1,58\ 10^3$	$3,99\ 10^3$	0	0	$4,52\ 10^2$	$1,49\ 10^4$	0	+	+	-
	0	$1,38\ 10^5$	0	0	$1,35\ 10^3$	0	0	$1,22\ 10^4$	$1,43\ 10^4$	0	+	+	+
R	1	$3,75\ 10^5$	0	0	$4,07\ 10^3$	$5,64\ 10^3$	0	0	$1,35\ 10^5$	0	-	-	-
	2	$3,50\ 10^5$	0	0	0	0	0	0	$4,44\ 10^2$	0	-	+	-
	3	$2,01\ 10^5$	0	0	0	0	0	0	$1,41\ 10^5$	0	-	-	-
	4	$2,00\ 10^5$	0	0	$2,23\ 10^3$	0	101,25	$3,89\ 10^2$	$3,57\ 10^4$	0	-	-	-
	5	$1,98\ 10^5$	0	0	0	0	540	0	$1,31\ 10^2$	0	-	-	-
HF	6	$1,56\ 10^5$	0	0	0	0	0	$5,88\ 10^3$	$3,91\ 10^6$	$3,93\ 10^2$	+	+	-
	7	$7,13\ 10^2$	0	0	0	0	0	54,3	$1,06\ 10^3$	0	-	-	-
	8	$7,27\ 10^5$	0	0	0	0	0	0	$4,10\ 10^2$	0	-	-	-
	9	$2,00\ 10^5$	0	0	0	0	0	0	0	0	-	-	-
	12	$1,80\ 10^5$	0	0	0	0	0	0	0	0	-	-	-
HP	13	$2,65\ 10^5$	$1,40\ 10^3$	0	0	0	0	75	$4,15\ 10^5$	0	-	-	-
	14	$6,46\ 10^5$	0	0	$1,31\ 10^3$	0	0	0	$2,89\ 10^2$	0	-	-	-
	15	$4,97\ 10^5$	0	0	0	0	0	23	$7,38\ 10^2$	0	-	+	-

R: regular diet; HF: high fiber diet; HP: high protein diet, S: sampling, VA: vermiform appendix, SR: sacculus rotundus, IM: ileum mucosa,
S0, S2, S3, and S7 did not yield positive fecal culture results. Tonsils, spleen, liver, cecal content, ileum, jejunum and muscle were negative by culture.
Tonsils and spleen were not determined by qPCR and liver, muscle, cecal content and ileocecal valve were all negative by this technique

Figure



Interpretation of the Multiple Correspondence Analysis (MCA) map: The right side contains close to positive results (positive Map culture) and high rates (high Map load in feces, high Map load in tissues, large lesion extension), whereas left side contains close to negative (negative Map culture) and low rates (low Map load in feces, low Map load in tissues, small lesion extension). Empty circles in the plane represent the categories of the measurements included in the MCA analysis being only the most representative ones were labeled. The relative position of the category points indicates the level of similarity or association between the categories. The closer the points are, the stronger the relationship between categories is. Diet was included in the map with illustrative purposes and all the individuals were also projected into the map. Relative positions of the subjects in this plane are represented by large circles in different colours (Diet R: orange, Diet HF: purple and Diet HP: green) and small rhombus in the same colours represents mean position of each diet group. Most Diet R individuals are on the right side of the Map implying a higher association with parameters that could represent a close to fulfilled infection status whereas Diet HF and Diet HP individuals lay on the left side of the map indicating a mild infection or a not properly achieved infection.

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