Metabolomic changes in *Mycobacterium avium* subsp. *paratuberculosis* (MAP) challenged Holstein–Friesian cattle highlight the role of serum amino acids as indicators of immune system activation

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Abstract

**Introduction** Paratuberculosis, commonly known as Johne’s disease, is a chronic granulomatous infection of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Clinical signs, including reduced milk yields, weight loss and diarrhoea, are typically absent until 2 to 6 years post exposure.

**Objectives** To identify metabolomic changes profiles of MAP challenged Holstein–Friesian (HF) cattle and correlate identified metabolites to haematological and immunological parameters.

**Methods** At approximately 6 weeks of age, calves (n = 9) were challenged with $3.8 \times 10^9$ cells of MAP (clinical isolate CIT003) on 2 consecutive days. Additional unchallenged calves (n = 9) formed the control group. The study used biobanked serum from cattle sampled periodically from 3- to 33-months post challenge. The assessment of sera using flow infusion electrospray high resolution mass spectrometry (FIE-HRMS) for high throughput, sensitive, non-targeted metabolite fingerprinting highlighted differences in metabolite levels between the two groups.

**Results** In total, 25 metabolites which were differentially accumulated in MAP challenged cattle were identified, including 20 which displayed correlation to haematology parameters, particularly monocyte levels.

**Conclusion** The targeted metabolites suggest shifts in amino acid metabolism that could reflect immune system activation linked to MAP and as well as differences in phosphocholine levels which could reflect activation of the Th1 (tending towards pro-inflammatory) immune response. If verified by future work, selected metabolites could be used as biomarkers to diagnose and manage MAP infected cattle.

**Keywords** *Mycobacterium avium* subspecies *paratuberculosis* · Metabolomics · Haematology · Amino acids · Immune response

1 Introduction

Paratuberculosis, commonly known as Johne’s disease, is a chronic granulomatous infection of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is commonly transmitted via the ingestion of infected faeces or colostrum (Sweeney, 1996) when calves are < 6-months of age (Windsor & Whittington, 2010). Following MAP exposure, infected calves enter a prolonged incubation period before the (sub)clinical stages of disease (Koets et al., 2015). Characteristic clinical signs, such as weight loss, diarrhoea and reduced milk yields (Whitlock & Buergelt, 1996), are often absent until 2 to 6 years post infection (Salem et al., 2013).

MAP contaminated cattle demonstrate an associated annual loss of £112.9/cow (€131.8/cow), including milk yield losses of £60.6/cow (€70.7/cow) (Barratt et al., 2018). Serum ELISA data indicate that UK and Irish dairy herds have similar herd prevalence’s of 34.7% (95% CI 27.6–42.5%) (Cook et al., 2009) and 31.5% (95% CI...
2.1 Study design, MAP culture and immunology assessments

The animal study was first described by Britton (2017) and was conducted in accordance with the Irish Health Products Regulatory Authority (Earlsfort Terrace, Dublin, Ireland) and approved by the UCD Animal Review Ethics Committee (UCD, Dublin, Ireland). The study was based on 55 HF calves (54 males and 1 freemartin) sourced from 2 farms with MAP seropositivity rates of ≤ 10%. All dams were MAP faecal culture negative and seronegative. Calves were housed indoors at the Central Veterinary Research Laboratory Farm (County Kildare, Ireland) for the duration of the study. To minimise cross contamination, the two groups were housed approximately 0.5 km apart and strict biosecurity measures enforced.

Calves were randomly assigned to the MAP challenge (n = 35) or control (n = 20) group. At approximately 6-weeks of age, the challenge group received an inoculation with 3.8 × 10^8 MAP bacterial cells (clinical isolate CIT003) on 2 consecutive days. Faecal MAP culture tests were performed at 3-, 6-, 9-, 12-, 16-, 20-, 24-, 28-, 31- and 33-months post challenge. Tissue culture was conducted upon euthanasia at either 12-, 24- or 33-months post challenge. Pathological evaluations assessed the ileum and ileocaecal valve, as well as the ileal and ileocaecal lymph nodes, for changes consistent with MAP infection. Blood samples were collected in EDTA tubes and measured for a range of haematology parameters, including monocytes (% and × 10^9/L) (Supplementary Table 1).

The CMI response was assessed 2-, 3-, 6-, 10-, 12-, 16-, 20-, 24-, 28-, 31- and 33-months post challenge using the Bovigam IFNγ release assay (IGRA) (Prionics AG, Schlieren-Zurich, Switzerland) following manufacturer’s instructions. IGRA results were determined by subtracting the mean delta optical density (ΔOD) of the phosphate buffered saline (PBS) stimulated wells from antigen stimulated wells. In line with IDEXX-criteria (Jungersen et al., 2022), results were classified as MAP-positive if the PPDb stimulated wells exhibited an ΔOD 0.999 higher than the PBS stimulated wells and if the PPDb stimulated wells divided by the mean of the PPDα wells was below 0.71.

The humoral immune response was assessed pre-challenge, 3-, 6-, 10-, 12-, 15-, 16-, 20-, 21-, 24-, 27-, 28-, 30-, 31- and 33-months post challenge using the Johne’s Identification and Verification ELISA (IDEXX laboratories Inc., Maine, USA) following manufacturer’s instructions. Initially, samples were tested using the Johne’s Identification ELISA, samples with a positive control ratio (S/P%) < 45% were classified as MAP negative. All samples ≥ 45% were retested with the Johne’s Verification ELISA. S/P% values between 45–54% were deemed inconclusive and S/P% values ≥ 55% were classified as MAP positive.

2.2 Untargeted metabolite fingerprinting by flow infusion electrospray ionization high resolution mass spectrometry (FIE-HRMS)

Sera from 9 MAP challenged and 9 control cows, which were euthanized 33-months post MAP challenge and sampled 3-, 6-, 9-, 12-, 15-, 21-, 24-, 28-, 31- and 33-months post MAP challenge were prepared as described by...
Beckman et al., (2008) with minor amendments. Samples were defrosted on ice, vortexed for 5 s and 200 µL was pipetted into 1520 µL pre-chilled solvent mix (methanol/chloroform [4/1]) containing 1 micro-spoon of glass beads. Samples were then vortexed for 5 s, shaken for 15 min at + 4 °C and kept at − 20 °C for 20 min. Following centrifugation at 22,000xg and 4 °C for 5 min, 100 µL of the serum supernatant was transferred into mass spectrometry vials along with 100 µL methanol/water [70/30]. For each sample, 20 µL were injected into a flow of 60 µL per minute water–methanol, at a ratio of 70% water and 30% methanol, using a Surveyor flow system into a Q Exactive plus mass analyser instrument with UHPLC system (Thermo Fisher Scientific©, Bremen, Germany) for high throughput FIE-HRMS. Data acquisition for each serum sample was done by alternating the positive and negative ionisation modes, throughout four different scan ranges (15–110 m/z, 100–220 m/z, 210–510 m/z, 500–1200 m/z) with an acquisition time of 2 min. Each serum sample was analysed in each scan range once. Time series analyses used false discovery rate (FDR) adjusted two-way ANOVA tests to identify m/z which significantly (p values < 0.05) differed between experimental classes. Partial least squares—discriminate analysis (PLS-DA) and variables of importance for the projection (VIP) scores (> 1) visualised the differences between the experimental classes. Major sources of variation were displayed using unsupervised hierarchical clustering analysis (HCA). Area under the curve (AUC) assessments based on sensitivity and specificity estimates was used to determine the accuracy of the target m/z as potential biomarkers. Random Forest (RF) was used as an alternative multivariate classification test. This uses a confusion matrix to estimate how often a given m/z variable would give an estimate of the classification error.

Significant m/z were identified based on accurate mass (5 ppm resolution) using the mummichog algorithm. All isotopes/adducts were considered. Metabolite set enrichment analysis (MSEA) using over representation analysis (ORA) was used to highlight key pathways and biological patterns. Correlation analysis between identified metabolites, immunology biomarkers and haematology parameters were performed using Pearson’s correlation coefficient.

2.3 Statistical analysis

Metabolomic data were analysed using MetaboAnalyst 4 (Chong et al., 2019). Data were subjected to interquartile range-based filtering, log_{10} transformations and Pareto scaling. Time series analyses displayed inconclusive results. Analysis of haematology parameters, including lymphocytes and monocytes, at 12-, 24- and 33-months post MAP challenge demonstrated no significant results (p > 0.05) (Supplementary Table 1).

3 Results

3.1 The MAP challenge study

The MAP challenge study has already been described by Britton (2017) and details are here included to aid comprehension of these follow-on metabolomic experiments. All cattle were faecal and tissue culture MAP-negative throughout the study. Post-euthanasia examinations of MAP challenged cattle highlighted a number of gross pathological changes, such as thickening of the ileum and enlarged ileo-caecal lymph nodes. However, all Ziehl–Neelsen (ZN) stained tissue smears were negative. MAP challenged cattle showed significantly higher IGRA (ΔOD) expression in response to purified protein derivative (PPD) from M. avium subsp. avium (PPDa) or M. avium subsp. paratuberculosis (PPDj) stimulation at 2-, 6-, 10-, 12-, 16-, 28-, 31- and 33- months post MAP challenge (Mann Whitney test; p < 0.05) compared to controls. Likewise, purified protein derivative from M. bovis PPD (PPDb) stimulation led to significantly higher IGRA (ΔOD) expression in infected cattle at 6-, 10-, 12-, 16-, 28-, 31- and 33- months post MAP challenge (Mann Whitney test; p < 0.05) compared to controls. Over the duration of the study, 7 MAP challenged and 2 control cattle were ELISA positive and a further 3 MAP challenged cattle displayed inconclusive results. Analysis of haematology parameters, including lymphocytes and monocytes, at 12-, 24- and 33-months post MAP challenge demonstrated no significant results (p > 0.05) (Supplementary Table 1).

3.2 Metabolomic changes

PLS-DA of the serum metabolite profiles derived from both negative and positive ionisation modes m/z exhibited some discrimination between MAP challenged and control cattle, albeit with overlapping of 95% confidence intervals, PLS-DA models 24-months post MAP inoculation are shown in Fig. 1. The sources of variation are displayed based on their VIP scores from PLS-DA models and were classified based on differences between control and MAP inoculated datasets. Supplementary Figs. 1 and 2 display the VIP scores (> 1) for the PLS-DA models 24-months post MAP inoculation. Nevertheless, the sources of variation between the experimental classes were identified based on time series two-way ANOVA (p < 0.05) and corrected for FDR. These assessments identified 25 significant metabolites (Table 1). Discrete metabolites showed either relative increases or decreases in MAP challenged samples compared to controls (Supplementary Figs. 3 and 4). Table 1 indicates that identified metabolites belonged
to various (sub)classes, although, there is a notable accu-
mulation of 6 metabolites from the amino acids, peptides
and analogues subclass. MSEA using ORA highlighted
the effect of MAP challenge on phosphatidylcholine bio-
synthesis and alanine metabolism in negative ionisation
mode (p < 0.05) (Supplementary Fig. 5) and arginine and
proline metabolism in positive ionisation mode (p < 0.05)
(Supplementary Fig. 6).

Of the 25 metabolites identified, all were significantly
affected by time and 12 out of 25 metabolites by MAP
challenge status *time (p < 0.05) (Table 1). Therefore,
the levels of the 25 metabolites derived from either nega-
tive (Fig. 2a) and positive ionisation modes (Fig. 2b)
are displayed over time using heat maps. Considerable
variation in m/z was observed, particularly at early time
points but there appeared to be improved consistency
from 15-months post MAP challenge. Within the negative
ionisation data, the abundance of metabolites involved
in nucleotide metabolism (deoxyadenosine and uridine),
the Krebs cycle (glyoxylic acid) and phosphatidylcholine
biosynthesis (α-phosphoethanolamine) decreased within
MAP challenged cattle. However, the majority of metab-
olite changes showed increases, particularly two keto
acids (maleylacetoacetic acid and 2-oxosuccinamate).
In contrast, in the positive ionisation data, levels of all
metabolites decreased in MAP challenged cattle, except
for malonyl-CoA. Interestingly, 4 out of 7 metabolites that
showed a decrease belong to the amino acids, peptides and
analogues subclass.

The specificities and sensitivities of each of the targeted
metabolites were assessed using AUC calculations (Supple-
mentary Table 2). Of the 25 identified metabolites, 5 metab-
olites (2-oxosuccinamate, 6-thioxanthine 5′-monophosphate,
acetaldehyde, biotin and (S)-ureidoglycolate) showed AUC
values of > 0.75 from 24-months post challenge. Other
metabolites detected using the positive ionisation mode
(3a,7a,12a-Trihydroxy-5b-cholestanolic acid, proline and
(S)-ureidoglycolate) exhibited discriminatory AUC val-
ues of > 0.90 within 3-months post challenge. However,
these AUC values dramatically decreased to < 0.60 within
6-months post challenge. Thus, AUC calculations suggested
that no metabolites were able to distinguish between experi-
mental classes at all stages of the study. These results were
consistent with the trends shown in Fig. 2a and 2b. Addi-
tionally, RF analysis reported class errors of 0.00 for both
MAP challenge and control cattle 33-months post challenge,
respectively.

We next assessed the ability of metabolomes to differen-
tiate between MAP challenge status and IGRA status. Sig-
ificant metabolite changes that differentiate between the
experimental classes are displayed using a heat map many
of which could not be unambiguously identified, and their
molecular formulae are provided (Fig. 3). There was lim-
ited overlap in the metabolites identified based on MAP or
IGRA status with only phenylacetylglycine, hippuric acid,
biotin and cytidine being common to both analyses. The heat
map showed that it was possible to differentiate between
MAP challenged and control cattle but not between control

Fig. 1 Partial least-squares discriminant analysis (PLS-DA) for MAP challenged and control cattle in the a negative ionization and b positive ionization modes 24-months post MAP-challenge. The light red and green ellipses represent 95% confidence intervals.
cattle which were IGRA positive or negative. These findings were reinforced by Pearson’s correlation analysis which showed poor correlation between identified metabolites and responses to PPDa, PPDb and PPDj stimulation (Supplementary Fig. 7). It should be noted that Britton (2017) showed significant differences between IGRA expression in response to PPDa and PPDj 2-, 6-, 10-, 12-, 16-, 28-, 31- and 33-months post MAP challenge.

Pearson’s correlation analyses were next used to examine the relationship between 25 identified metabolites, the CMI and humoral immune responses, as well as the 17 haematological parameters examined. Of the 525 analysed correlations, 28 were positively correlated (correlation coefficient > 0.4) and 26 were negatively correlated (correlation coefficient < -0.4). Of the 54 observed correlations, 53 were between identified metabolites and haematological parameters (Supplementary Fig. 8). As also shown by Britton (2017), no significant differences were observed within the haematology parameters of MAP challenged and control cattle. Nevertheless, most of the haematology parameters showed significant correlations with 1 or more identified metabolites. Monocytes (%) and MCH (pg) were most frequently correlated to metabolites, displaying 11 and 6 correlations, respectively. (Supplementary Table 3). Keto acids (2-oxyosuccinamate and maleylacetoacetic acid) showed the most significant negative correlation (Supplementary Fig. 7 and Fig. 4a), whilst glyoxylic acid and (S)-ureidoglycolic acid from the carboxylic acids and derivatives class displayed the strongest positive correlation to monocytes (Supplementary Fig. 9 and Fig. 4b).

| Table 1 | Metabolites differentiating between MAP challenged and control cattle |
|---|---|
| **Class** | **Subclass** | **Metabolite** | **Ionization mode** | **MAP challenge** | **Time** | **Interaction** |
| Benzene and substituted derivatives | Benzoic acids and derivatives | Hippuric acid | Negative | 3.74 × 10^-2 | 2.12 × 10^-18 | 5.54 × 10^-3 |
| Biotin and derivatives | – | Biotin | Negative | 1.85 × 10^-3 | 4.72 × 10^-6 | 7.83 × 10^-3 |
| Carboxylic acids and derivatives | Amino acids, peptides, and analogues | (S)-Ureidoglycolic acid | Positive | 1.09 × 10^-2 | 5.17 × 10^-16 | 3.06 × 10^-1 |
| | | 3-Sulfinoalanine | Positive | 4.18 × 10^-2 | 3.67 × 10^-2 | 9.39 × 10^-2 |
| | | Citrulline | Positive | 1.85 × 10^-2 | 3.51 × 10^-4 | 5.10 × 10^-2 |
| | | Methionine | Negative | 4.74 × 10^-2 | 1.36 × 10^-4 | 5.35 × 10^-3 |
| | | Proline | Positive | 3.61 × 10^-2 | 1.25 × 10^-17 | 8.34 × 10^-3 |
| | | Phenyacetyleglycine | Negative | 2.85 × 10^-2 | 1.57 × 10^-11 | 1.19 × 10^-6 |
| Dizaines | Carboxylic acids | Glyoxylic acid | Negative | 2.66 × 10^-2 | 1.31 × 10^-3 | 2.74 × 10^-1 |
| | Pyrimidines and pyrimidine derivatives | 6-Thioxanthine 5’-monophosphate | Negative | 3.68 × 10^-3 | 2.00 × 10^-17 | 1.56 × 10^-7 |
| Fatty acyls | Fatty acids and conjugates | Arachidic acid | Negative | 3.02 × 10^-2 | 2.31 × 10^-15 | 6.43 × 10^-4 |
| | Glycosylglycerols | Galactosylglycerol | Positive | 1.55 × 10^-2 | 6.80 × 10^-12 | 4.88 × 10^-3 |
| Keto acids and derivatives | Medium-chain keto acids and derivatives | Maleylacetoacetic acid | Negative | 3.09 × 10^-3 | 2.48 × 10^-8 | 4.78 × 10^-6 |
| | Short-chain keto acids and derivatives | 2-Oxosuccinamate | Negative | 1.55 × 10^-2 | 4.75 × 10^-6 | 1.50 × 10^-1 |
| Organic phosphoric acids and derivatives | Phosphate esters | O-Phosphoethanolamine | Negative | 4.89 × 10^-2 | 1.10 × 10^-6 | 6.89 × 10^-2 |
| Organonitrogen compounds | Quaternary ammonium salts | Phosphorylcholine | Negative | 2.32 × 10^-2 | 2.52 × 10^-13 | 4.88 × 10^-2 |
| | Carbohydrates and carbohydrate conjugates | Xylose | Positive | 3.61 × 10^-2 | 3.10 × 10^-7 | 4.27 × 10^-1 |
| | | N-Acetyleuraminic | Negative | 1.19 × 10^-2 | 4.61 × 10^-4 | 9.37 × 10^-1 |
| | | 4-Aminobutyraldehyde | Negative | 3.68 × 10^-2 | 1.43 × 10^-3 | 2.89 × 10^-1 |
| | | Acetaldheyde | Negative | 4.94 × 10^-4 | 3.29 × 10^-12 | 3.73 × 10^-5 |
| | | Malonyl-CoA | Positive | 3.16 × 10^-2 | 7.04 × 10^-18 | 6.44 × 10^-3 |
| Purine nucleosides | Purine 2’-deoxyribonucleosides | Deoxyadenosine | Negative | 2.32 × 10^-2 | 2.27 × 10^-3 | 3.29 × 10^-2 |
| Pyrimidine nucleosides | – | Cytidine | Negative | 5.89 × 10^-3 | 4.49 × 10^-4 | 3.51 × 10^-3 |
| | | Uridine | Negative | 4.89 × 10^-2 | 1.72 × 10^-4 | 1.00 × 10^-4 |
| Steroids and steroid derivatives | Bile acids, alcohols and derivatives | 3a,7a,12a-Trihydroxy-5b-cholestanolic acid | Positive | 3.61 × 10^-2 | 9.05 × 10^-7 | 4.37 × 10^-3 |
Blood from MAP challenged cattle stimulated with PPDa exhibited positive correlation with phosphocholine, but blood stimulated with PPDb or PPDj, or serum ELISA data showed either no, or only a weak correlation with identified metabolites (Supplementary Fig. 7). Interestingly, 6-thioxanthine 5'-monophosphate and phenylacetylglycine exhibited peaks 28-months post MAP-challenge (Fig. 5a and 5b). Despite showing a weak overall correlation to PPDa, PPDb and PPDj, this peak coincides with the 2nd peak in IGRA (ΔOD) expression in response to PPDa, PPDb and PPDj stimulation (Supplementary Fig. 10).

4 Discussion

Paratuberculosis is a chronic disease that represents an ongoing challenge to farmers. The management of MAP will be improved if the disease is better understood and, in this, the application of ‘omic approaches have a major role to play. However, metabolomics has only rarely been applied in MAP research. This stated, metabolomic approaches have been used to show the impact of MAP on energy, lipid and protein metabolism within naturally and experimentally infected cattle (De Buck et al., 2014; Tata et al., 2021). We
sought to extend such studies by examining the effects of experimentally challenging cattle with MAP with a view to providing new clinical insights and possibly identifying novel metabolite biomarkers. To provide additional insights and to provide wider context, we sought to correlate the key, identified metabolites with cell mediated and humoral immune responses, as well as haematological parameters. FIE-HRMS does not utilise prior chromatographic separation and this allows a wide dynamic range of metabolite content to be measured with a high sensitivity. This, coupled with a high resolution (3 ppm) that facilitates metabolite identification, allows the chemical content of a sample to be measured with a high sensitivity.

This stated, other tests results could be linked with MAP infection, including IGRA expression in response to PPDa, PPDb and PPDj stimulation being significantly higher in MAP challenged cattle. Time series two-way ANOVA (p < 0.05) analyses corrected for FDR indicated how identified metabolites significantly changed over the duration of the study (Table 1). However, within the experimental classes, as shown by HCA, considerable variation was observed at the early time points, but this reduced from 15-months

**Fig. 3** Significant metabolite changes (negative ionization model) differentiating between MAP challenge status and interferon-y release assay IDEXX criteria MAP interpretation results between 24 and 28-months post MAP challenge
post MAP challenge (Fig. 2). Considering that male bovine puberty occurs between 37 and 50 weeks of age (Rawlings et al., 2008), sexual maturity is unlikely to be a major driver of metabolite fluctuations in this experiment. However, HF steers continue to grow until a liveweight of ~620 kg is achieved at approximately 24 months of age (O’Riordan et al., 2019). Additionally, the impact of diet on the bovine metabolome is well-documented (Yang et al., 2018). Therefore, a mixture of growth and dietary factors are likely to contribute to metabolite variation up to 15-months post MAP challenge. Similarly, a longitudinal trial conducted by De Buck et al., (2014) of MAP infected HF heifers and age-matched controls sampled over 17-months noted that key variables were significantly affected by time. These metabolomic changes were suggested to reflect development and dietary changes.

4.1 MAP exposure is typified by amino acid and phospholipid changes in the serum metabolome.

Considering the discriminatory metabolites, it was notable that amino acids, peptides, and analogues subclass (class—carboxylic acids and derivatives) showed prominent changes following MAP challenge. Methionine and phenylacetyl-glycine levels increased, whilst (S)-ureidoglycolic acid, 3-sulfinoalanine, citrulline and proline decreased (Fig. 2). Although these metabolites have not been previously associated to MAP, the effect of MAP on amino acid levels is well-documented. Thus, De Buck et al., (2014) reported significant increases in amino acids; tyrosine, threonine, isoleucine, leucine and asparagine, within MAP infected cattle. Likewise, Tata et al., (2021) found increased creatine, 2-oxosuccinamate and maleylacetoacetic acid as well as glyoxylic acid and ureidoglycolic 33-months post MAP challenge.
creatinine and tryptamine levels within MAP infected and infectious cattle, compared to controls. These changes were suggested to be linked to a reduced absorptive capacity due to MAP induced inflammation (De Buck et al., 2014). However, this does not accord with our observations as only 3 MAP challenged cattle included in the metabolomic analysis showed thickening and corrugation of the ileum, and none exhibited positive ZN stains (Britton, 2017). It may be that a reduced absorptive capacity is not the cause of altered amino acid metabolism.

Other amino acid changes could be more easily associated with the possible effects of MAP infection. MSEA using ORA showed that alanine metabolism was significantly affected by MAP challenge (Supplementary Fig. S5). Biotin was significantly increased but glyoxylic acid was significantly decreased in MAP challenged cattle (Fig. 2a). These results suggest that alanine was converted to pyruvic acid by NAD(H) dependent L-alanine dehydrogenase (ALD), in conjunction with glyoxylic acid being converted to glycine by glycine dehydrogenase (Giffin et al., 2011). Pyruvic acid would then be converted to oxalacetic acid, via pyruvate carboxylase (using biotin as a co-factor), to feed the citric acid cycle (SMPDB, 2019). Previous studies have demonstrated the oxidative deamination of alanine to pyruvate to aid the growth of *Mycobacterium tuberculosis* (Giffin et al., 2011), *Mycobacterium bovis* Bacillus Calmette-Guérin (*M. bovis* BCG) (Chen et al., 2003) and *Mycobacterium smegmatis* (Feng et al., 2002). Therefore, these changes in host sera may be indicative of MAP metabolism within challenged cattle.

MSEA using ORA also suggests that phosphatidylcholine biosynthesis was significantly affected by MAP challenge (Supplementary Fig. S5). This indicated that o-phosphoethanolamine and N-trimethyl-2-aminoethylphosphonate (also known as phosphorylcholine) differed following MAP inoculation. Reduced levels of phosphocholine-containing lipids, such as phosphatidylcholines, choline plasmalogens, and sphingomyelins have previously been identified as features of MAP infected cattle that were displaying clinical signs (Wood et al., 2018). Our data suggests that MAP challenges induce minor changes in phosphatidylcholine biosynthesis within MAP challenged cattle during the incubation period.

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**Fig. 5** Box and whisker plots of metabolites which display reduced overlapping between groups, MAP challenged and control cattle, from 21 months post MAP challenge. Blue boxplots = MAP challenged cattle, green boxplots = control cattle. (Color figure online)
but major shifts do not develop until the clinical stage of infection. Furthermore, our correlation analyses indicated a positive correlation between PPDa stimulated blood and phosphorylcholine. Both PPDa stimulated blood and phosphorylcholine showed a two-peaked pattern, starting out at a high level but reducing 21 to 24-months post MAP challenge before increasing again. (Fig. 2a; Supplementary Fig. 10). PPDa antigens highlight Th1 immune responses (mostly pro-inflammatory) and IFN-γ has been shown to increase phosphatidylcholine hydrolysis and phosphorylcholine production in human HeLa-S3 cells (Pfeffer et al., 1990). Thus, elevated phosphorylcholine expression is suggestive of Th1 immune response activation.

4.2 The MAP-challenged cattle metabolome may reflect immunological changes

To develop our understanding of the biological relevance of the metabolomic differences following MAP infection, we correlated these with key haematological data as provided by Britton (2017). This indicated correlations (−0.4 < correlation co-efficient > 0.4) between 53 of the metabolites and haematology parameters, particularly MCH and monocytes (Supplementary Table 3; Supplementary Fig. 8). At the onset of infection, monocytes are recruited to the affected tissue where they differentiate into macrophages or dendritic cells (Yang et al., 2014). Macrophages play important roles in destroying MAP-infected tissues and therefore preventing disease progression (Jenvey et al., 2019). Interestingly, alanine metabolism, which was significantly affected by MAP challenge (Supplementary Fig. 5), is altered in M2 macrophages (Abuawad et al., 2020) and the alanine-associated metabolite, glyoxylic acid was correlated with monocytes levels (Supplementary Fig. 8). Th1 cells secrete cytokines such as IFN-γ, to promote macrophage activation and cytotoxic T cell proliferation and MAP elicited changes within the alanine metabolism are likely to contribute to this immune response through M2 macrophage recruitment. Beyond alanine metabolism, monocytes displayed this immune response through M2 macrophage recruitment in which PPDA antigens highlights Th1 immune responses (mostly pro-inflammatory) and IFN-γ has been shown to increase phosphatidylcholine hydrolysis and phosphorylcholine production in human HeLa-S3 cells (Pfeffer et al., 1990). Thus, elevated phosphorylcholine expression is suggestive of Th1 immune response activation.

5 Conclusion

Metabolomic analysis of MAP challenged and control cattle between 3- and 33-months post MAP challenge using untargeted FIE-MS highlighted metabolites indicative of MAP exposure. In total, 25 metabolites which were differentially accumulated in MAP challenged cattle were identified, including 20 which displayed significant correlation to haematological parameters. The targeted metabolites suggest that MAP induces changes in amino acid and phosphorylcholine levels which are suggestive of a Th1 immune response. Future work could include identifying these metabolites in naturally MAP infected cattle and tissue or faecal culture positive cattle, as well as measuring their accumulations in M. bovis infected cattle to verify their specificity to MAP.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11306-022-01876-w.

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Author contributions BM, SG and LM conceived and designed research. MB developed the methodology. ET conducted experiments well as measuring their accumulations in M. bovis infected cattle to verify their specificity to MAP.

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Declarations

Conflict of interest Emma N. Taylor, Manfred Beckmann, Bryan K. Markey, Stephen V. Gordon, Glyn Hewinson, David Rook, Luis A. J. Mur declares that he has no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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