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Zonulin as a Preliminary Biomarker of Lung Permeability Among Diseased Calves: Cohort Study

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ZONULİN, almost well recognized protein, not solely influence the integrity of intercellular connections int the gut, but also could be capable of modulating lung permeability. It is best known as biomarker/regulator of gastrointestinal tight gap junctions. On the other hand cumulative evidence is growing regarding gut-lung axis also in cattle. The purpose of this present cohort study was to establish the relationship between circulating zonulin levels and severity of respiratory problems among disease calves upon referral at a milk-fed veal facility, 20 calves with a diagnosis of bovine respiratory disease were scored for calf respiratory scoring chart previously identified and well designed. Scores >5 were identified as active disease (severe pneumonia), whereas scores <5 were denoted as exposure (without severe pneumonia). Commercially available specific Bovine Zonulin ELISA test kits were used. Ethical guidelines were strictly followed up with written owner consent was available for all calves enrolled. The mean zonulin levels (ng/mL) healthy calves, calves without severe pneumonia (Score <5) and calves with pneumonia (Score >5) were detected as 21.03 ± 10.57, 39.53 ± 11.90 and 64.69 ± 17.98, respectively. There was statistically significant alterations in calves with severe pneumonia in contrast to healthy calves ($p<0.05$). As preliminary findings of the present reseacrh zonulin is not solely limited to expression of intestinal inflammation and thus circulating zonulin levels should be interpreted in possible lung injury (and or lung permeability) in correlation with disease activity on field conditions for prognosis and available treatment practices.

Keywords: Zonulin, Lung Permeability, Diseased Calves.

Introduction

The novel terminology involving different titles/ explanation denoted as 'the gut-lung axis' (Fig. 1): i) Intestinal microbiota and inflammatory lung disease, ii)The dialogue among gut microbiota and lung, iii)Gut microbiota and respiratory diseases, iv) Intestinal health and respiratory problems' [1-4] prompted us to perform this study in which novel biomarkers for detecting etiology, pathogenesis and possible treatment outcomes are warranted as because traditional treatment of bovine respiratory diseases are being non-efficacious and treatment failures are frequently reported in our landscape.

Novel researches indicated the intestinal microbiota influenced on the lungs (so called "gut-lung axis)," in which modulated via inflammation, microbiota derived products translocation [even if the barrier integrity is compromised or via mesenteric lymphatic route between gut-lung axis] move to systemic circulation and influence lung immun respond [5]. A crosstalk among the gut and lung microbiota as detected, denoted that alterations in one compartment might influence the

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other compartment (in association with microbial composition/ function) [5,6]. The ecological causal factors of the lung microbiota (immigration/ elimination/regionalgrowth circumstances) alter during acute/chronic lung disorders (ie. chronic obstructive pulmonary disease and lung cancer) [7,8], which may also the case in bovine respiratory disease among calves. In the present study, thus, the purpose was to analyze zonulin levels as a probable regulator of lung permeability among calves with bovine respiratory disease (non-classified to etiological background).

Material and Methods

Demographic Field Data

This cohort study was performed (as multidisciplinary collaboration of veterinary surgeons whom took responsibility for receiving/ taking blood samples) through a milk-fed veal facility in Aegean Region of Turkey. All diseased calves [monitored within the clincal signs diffuclty in breathing, rapid/shallow breathing, coughing, depression, nasal discharge, and anorexia] enrolled at the present study througly participated with written owner consent. The present study was approved by the local ethic committee of Aydin Adnan Menderes University-HADYEK report no: 64583101/2021/146 (27.10.2021). During trial ethical guidelines were entirely followed up within the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html) were carefully elucidated throughout all study with humanity. Demographic data was limitedly given herein as because of this was a field study the calves were from both sexes (19 female, 11 male), at the age of 19 to 76 days old from various breeds. All blood samples were withdrawn on 17-20 February 2023.

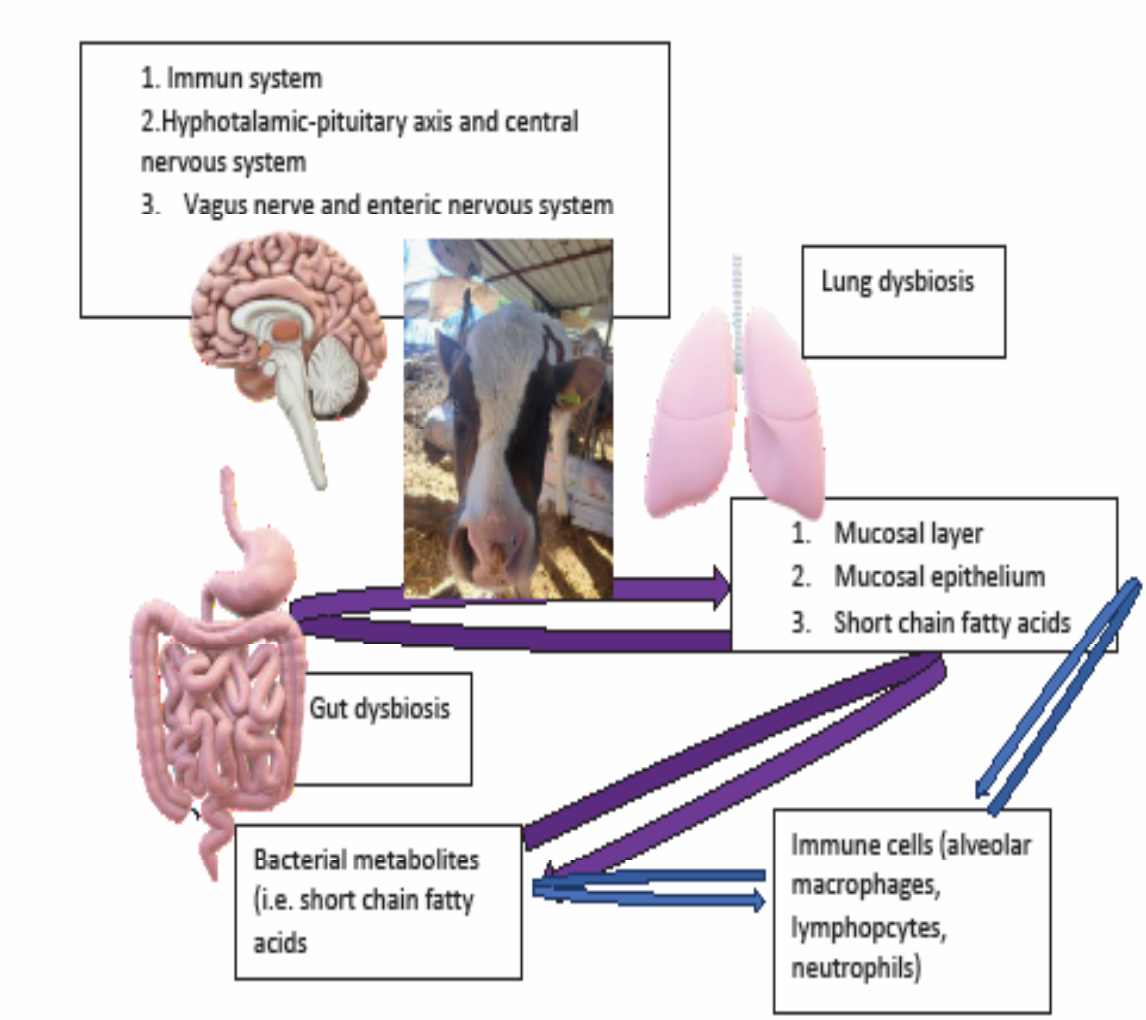


Fig. 1. Gut-lung axis depicting [self-drawn, but adopted from Welch et al. [9] and Shah et al. [10].

Sampling and Field Research

In a total of 30 calves enrolled, 1.5 ml blood was withdrawn (via field veterinary surgeon) from jugular vein into anticoagulated tubes. There afterwards obtained samples were entirely sent to laboratory work. Commercially available Bovine Zonulin ELISA test kits (MyBiosource ELISA kits, USA) (Fig. 2) were purchased (Turkish side distributor RDA Group, Istanbul). Performed and used ELISA methodology (Fig. 2) was similar to prior articles by the present author [11,12]. Briefly assay type was Sandwich ELISA, with a detection range of 100 ng/ml-1.56 ng/ml [sensitivity of 0.5 ng/ml]. Commercially available ready-to-use microwell, strip plate ELISA Kits were deemed available in an attempt to analyze existence of the Zonulin, ELISA Kit target analytes in sera samples. Available Sandwich ELISA established biochemical metholodogy for commercially available kit was based on Zonulin antibody-Zonulin antigen interaction (immunosorbency) and an HRP colorimetric detection unit for determination of Zonulin antigen targets in samples.. Fecal scoring system adopted from Graham et al. [13] were shown above in table 1.

Laboratory diagnostics and scoring system

As the first author of this manuscript is an agriculture engineer and from department of zootechnics, a quick, easy and if possible, non-invasive scoring was necessary. Calf respiratory scoring chart previously identified and well-designed [14] were preferred based on assessment of calves at 4 different categories [i.e., nasal discharge, cough, rectal temperature and ear/eye]. Scores obtained from 4 category are summed up to combine with a total respiratory score. Even if total respiratory score is >5 (which was also the cutoff point for the present study) the calf is diseased and treatment necessary. Score under 5 did not mean the calf is not diseased however at the present study scores above 5 has been denoted as pneumonia (active disease) which was briefly explained also in results section. Furthermore on first occasion, a minimal-volume (thirthy mL saline) nonendoscopic bronchoalveolar lavage was performed in 7 standing (without any sedation) calves similar to previous methodlogy [15]. Besides quick thoracic ultrasonography [16-18] (was preferred as an on-farm and rapid point of care methodology for supportive diagnose clinical/subclinical pneumonia in dairy calves. Consolidations ≥1 cm were similarly [18] determined as lobular pneumonia and to those of calves with ≥3 cm as lobar pneumonia. On the other hand subclinical pneumonia denoted no presenting severe clinical signs, but consolidations ≥1 cm on ultrasonography) whereas clinical pneumonia described both clinical signs and consolidations ≥1 cm observed on ultrasonography [19, 20].

Results

In the present study serum (mean ±standard error) zonulin levels (ng/mL) among healthy calves (21.03 ± 10.57) were significantly lower than calves without severe pneumonia (39.53 ± 11.90) and calves with pnemonia (64.69 ± 17.98) ($p<0.05$) (Table 1, Fig. 3 and 4).

Fig. 2. Commercially available test kits and ELISA plate as shown, were deemed valid in the present study.

TABLE 1. Zonulin levels among healthy calves and disease calves [score<5 in calves without severe pneumonia and Score >5 calves with pneumonia]. Different letters showed statistical significance.

	Zonulin (ng/mL)
Healthy Calves	21.03 ± 10.57^{a}
Calves without severe pnemonia (Score <5)	39.53 ± 11.90^{ab}
Calves with pnemonia (Score >5)	64.69 ± 17.98^{b}

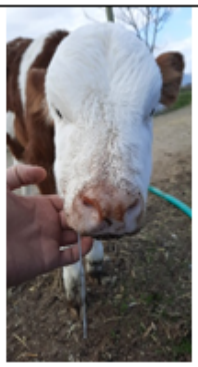
	Healthy calves (n=10)	Calves with a score <5 (n=10) without severe pneumonia (-)	Calves with a score >5 (n=10) Pneumonia (+)
Zonulin levels (ng/mL) (min*max)	10.12-43.40-	23.12-67.40	30.75-85.47
			

Fig. 3. Calf respiratory scoring chart showing zonulin levels, scoring system involvement [14] and enrolled cases (selected from each group).

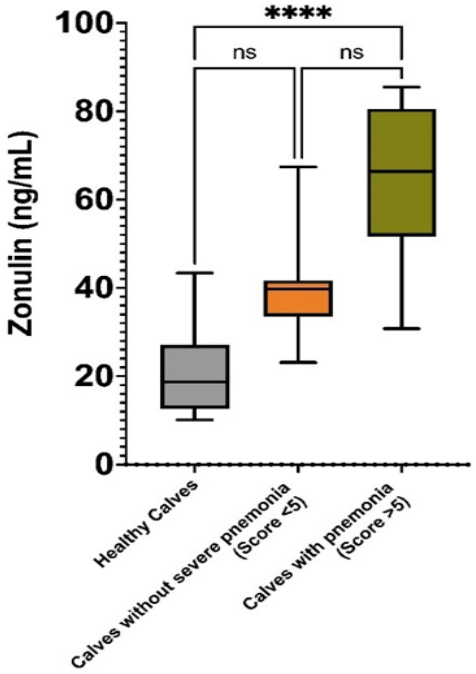


Fig. 4. Boxplot analytes regarding healthy calves and other relevant calves with or without pneumonia based on scoring.

Scoring system along with classification attended to the groups

In a total of 3 groups:

Group I: Healthy calves (n=10),

Group II: Calves with a score <5 (n=10) without severe pneumonia (-) [denoted as subclinical pneumonia (no clinical signs, but consolidations ≥1 cm on ultrasound), and Group III: Calves with a score >5 (n=10) pneumonia (+) [determined as clinical pneumonia (both clinical signs and consolidations ≥1 cm] as shown in fig. 3. scores and min-max. serum zonulin levels were shown above. On the other side boxplot analytes were presented at fig. 4 and statistical analysis at table 1 below. Diseased group of calves in group III were all presenting lobular pneumonia.

Discussion

Freshly, growing and arousing interest for better understanding the composite collegial relationship existing among gastrointestinal system microbial population and the host. Specifically gut microbiota interconnect through entire body along with microbiota-gut-organ axes. The metabolites existed within the resident microbiota at the gastrointestinal tract send signals to different organs, influencing the immune respond and physiology of the host [22]. The latter interplay among resident microbes and the immune system modulates organs at the host and there exists an "axis" capable of signalization [23]. Selected parts of these axes in cattle composed recognized gut-brain axis and gut-lung axis [24], which was also the subject of the present study.

Prior to COVID-19, zonulin-dependent intestinal tight junctions permeability has been recognized through chronic inflammation [25]. Even if zonulin is released and intestinal barrier function could be lost along with bacterial translocation, triggering immune respond within pro-inflammatory cytokine existence, in which all could worsen intestinal permeability [26]. On the other hand up-/down-regulation of zonulin was detected extra-intestinally at the lungs and brain [27,28]. Whilst the larger part of studies inspecting the mechanisms of tight junction regulation is almost targeted on intestinal epithelium, whereas a prior research [28] and the present study demonstrated that zonulin may also be involved in the regulation of epithelial and/or endothelial barriers (probably) in the lung. The latter hypothesis was supported by our findings that in the present study. According to our results healthy calves presented zonulin levels (ng/mL) (21.03 ± 10.57) lower that calves without severe pneumonia (Score <5) 39.53 ± 11.90 and calves with pneumonia (Score >5) 64.69 ± 17.98 (Fig. 3 and Table 1). There was statistically significant alterations in calves with severe pneumonia in contrast to healthy calves ($p<0.05$).

Aforementioned data should be discussed in depth as because mechanisms behind should be highlighted. Given capillary leak and epithelial barrier integrity loss presented features of acute lung injury and acute respiratory distress syndrome [28], there exists few knowledge regarding tight junction participation and how it could influence inflammatory cascade. During acute lung injury, large-scale demolition of tight junction proteins (ZO-1, claudin-5, claudin-3, and occludin) consequently cause leaky lung [28]. On the other side during acute lung injury and acute respiratory distress syndrome, lung permeability to a great extent elevated in relationship with , infectious /inflammatory/ toxic or traumatic etiology, could be capable of causing edema of the pulmonary parenchyma, capillary leak and subsequent respiratory failure [29]. Zonulin to this extent, is capable of detecting leakage, and as a regulator of lung permeability [28] was detected in the present study to those calves with respiratory disease. Furthermore given respiratory disorders as a significant problem in bovine neonates, the earlier determination [14] should have helped recovery, survival and prognosis in the newborn calf. Zonulin to this extent, could participate as an earlier detection biomarker denoting lung permeability, which is in capable of directing veterinarians for earlier treatment protocols. Our next study would thus be aimed to this issue.

Conclusions

Regarding evidenced based medicine this study has the potential to add scientific proof, in which zon ulin, not only limited to its tight junction biomarker dedicated to gastroenterology, could thus also be used in pulmonary disorders. As suggested with this study zonulin could be useful as a preliminary biomarker for testing lung permeability. Given bovine respiratory disease larger calf populations with categorical disease classification are warranted for better understanding 'leaky lung'. Our next study would

probably involve cross matched other pulmonary biomarkers and its correlation with zonulin levels in calves with pneumonia.

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Conflicts of interest

The authors declared competitive interest. We are open for collaborations.

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